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THE GENETIC FACTOR IN POPULATION ECOLOGY

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INTRODUCTION

The ecological problem of populations has to do with the numbers of animals and what determines these numbers. The genetical problem of populations has to do with the kind or kinds of animals and what determines kind. These two disciplines meet when the questions are asked, how does the kind of animal (i.e., genotype) influence the numbers and how does the number of animals influence the kind, i.e., the genetical composition of the population? These questions are as much ecological as they are genetical.

GENETIC AND PHENOTYPIC PLASTICITY

Chapman (1928) made a fundamental contribution to animal ecology when he introduced the important idea that the ecological characteristics of a species can be measured and regarded as constants in the sense in which chemical substances can be defined precisely in terms of certain constant characteristics. Such were birth-rate, death-rate, rate of development and so on. He considered these as characteristics of the species in the same way in which a taxonomist might regard certain morphological features as characterizing the species. Chapman had the idea that one day the ecologist would have at his disposal a table of ecological constants for the different species he worked with. Since Chapman's day some ecologists have attempted to measure quantitatively these qualities of ecological importance. The initial problem was to define them precisely. We need only recall Chapman's concept of "biotic potential" and the changes this concept has undergone, to appreciate the time and thought that was necessary to transform this into something that was both measurable and meaningful. The concept of dispersal is another example. At one time dispersal was the subject of anecdotes and little more. Today it can be studied precisely with the tools of statistics and chemistry.

It was perhaps fortunate that the attention of ecologists was directed by Chapman toward the end of defining more precisely ecological characteristics of animals, before they became confused by the fact that such qualities are not really constants at all. They are probably not constant even for a particular genotype, particularly if the genotype has phenotypic plasticity,

by which is meant the ability of the genotype to survive and multiply in a wide range of environments. However, populations consist of an array of genotypes and we now know, at least for quite a number of species, that this array changes both in space and in time. Instead of thinking of ecological characteristics of species as constants, we have been forced by genetics to think of them as fluctuating between certain values or even drifting in time to different mean values. Likewise we have to think of a transect in the spatial distribution of the species as revealing a spectrum of values corresponding to a spatial genetic spectrum. From a long term evolutionary point of view this characteristic of genetic plasticity has made evolution or transformation of species possible. From a short term ecological point of view it enables a species to live in a wide range of environments. And in a changing environment it enables the species to cope with change by changing itself. A change in environment may mean a new "ecological opportunity" for a species which has genetic plasticity. Genetic plasticity and phenotypic plasticity are two ways in which species are adapted to survive and multiply in a wide range of environments or, which amounts to the same thing, are adapted to cope with a change in environment. Since change is a more characteristic feature than stability in environments, these two sorts of plasticity are of fundamental significance for the ecologist to recognize and study.

The basis of genotypic plasticity is diversity of genotypes on which selection can operate. As a mechanism of adaptation to changing environment it can only be effective when the length of generation is shorter than the time within which the environment changes. For example, it is of no use to an elephant as an adaptive mechanism to seasonal change in the environment. But it is for Drosophila which can complete several generations in a season. On the other hand, it could presumably be of value to the elephant in adapting the species to long term changes in its environment such as must have occurred in the evolution of elephants.

Adaptation through genotypic plasticity is only possible when there is considerable genetic variability available at all times. The source of such variability is twofold: mutation and, in sexual organisms, recombination of genes. Recurrent mutation is the main source of genetic variability in bacteria, algae and protozoa. But mutation rates are, for the most part, so low that the effectiveness of mutation on its own has been questioned for all but such organisms as these which multiply at a prodigious rate. A high rate of increase combined with intense selection can lead to rapid change despite relatively low mutation rates. With sexual organisms mutation rate is reinforced with the element of stored variability which means that there is a reserve of variability over and above that which mutation alone can provide. This important aspect of adaptation in sexual organisms has been discussed in detail by Dobzhansky (1951) and Waddington (1957) and others, to whom reference should be made for details. Suffice it to indicate here the main genetic mechanisms involved in stored or concealed variability.

(a) Genes which have no phenotypic expression will be stored in the gene pool since it is the phenotype which is selected. Two mechanisms are involved, dominance and canalizing selection. Recessive genes concealed by dominant alleles will only become expressed phenotypically when homozygous. A tremendous store of recessive genes is concealed in heterozygotes. This was first shown by Tschetwerikoff in 1927 (see Dobzhansky, 1951) in natural populations of *Drosophila melanogaster* and has been amply confirmed for numerous other species by Dobzhansky (1951) and others since. Canalizing selection tends toward a stability of the phenotype through selection of genes which make the organism insensitive to abnormal environments (Waddington, 1957). Genes for a canalized character will inevitably be stored.

(b) Polygenic inheritance favors stored variability (Mather, 1941, 1943). This variability becomes released in linked polygene complexes when crossing over occurs and such released variability can be adaptive when environment changes.

(c) Heterosis or superiority of heterozygote over the homozygotes. This is the essential condition for balanced polymorphism in Mendelian populations. If a mutant produces a heterotic heterozygote, natural selection will retain this mutant in the population even if the homozygote is lethal. This mechanism has been extensively studied by Dobzhansky (1951) and his colleagues in Drosophila populations which exhibit polymorphism in chromosomal inversions. Heterosis is the mechanism by means of which a diversity of chromosomal inversions is maintained in the population. This diversity adapts the species to a wider variety of environments than would otherwise be possible (Dobzhansky et al., 1950). The existence of heterosis of single pairs of genes is more difficult to establish. The superiority of the heterozygote carrier of sickle-cell anaemia in malarial environments may be such an example (Allison, 1955). Other examples may occur in man (Dobzhansky, 1958) and Drosophila (Wallace, 1958).

A striking example of the release of stored variability with change in environment is Waddington's (1957) experiment in which he finds that in some flies of *Drosophila melanogaster* a cross-vein in the wing is missing when they are reared at a high temperature. The abnormality does not occur at normal temperatures. By selecting for cross-veinless flies at high temperature, Waddington found that the character was genetically determined. In his selected stock the character appeared irrespective of whether they were raised at low or high temperatures. The capacity to respond to high temperature in this way is resident in the normal unselected flies. The character cannot however be selected until environment favors its phenotypic expression. From this experiment Waddington argues for the genetic assimilation of adaptive characters induced by environment. One need only suppose that cross-veinlessness was adaptive at high temperatures to see his point. The capacity for response to environment is inherited. All the environment does

is to cause the genes already present, but concealed, to be expressed. Once expressed the new phenotypes can be selected. This mechanism would be far more effective in adapting a species to changed environment than reliance on random recurrent mutation alone.

Genetic plasticity and diversity of genotype is of no advantage in an environment which is constant in its properties except as an insurance against possible change in the future. Lewontin (1958) has pointed out that in a constant environment if a homozygote arose which was superior or equal in fitness to the heterozygote then fixation of that allele would result. This he claims to have demonstrated in a population cage of *Drosophila pseudoobscura* which initially contained two chromosomal inversions but after 1000 days the population was nearly homozygous for one inversion. The homozygous population is the "narrow specialist"; the heterozygous population may be less fit in any one environment but this is the price it pays for being able to live in a variety of environments by virtue of the diversity of its genotypes.

A balance between flexibility and stability of the genetic composition of a population is attained by a variety of genetic mechanisms. Chromosomal inversions are one way of tying up blocks of genes which have been proved to be of adaptive value in selection. Inversions suppress crossing over with consequent reduction in variability. This would be advantageous in some environments. But where the environment is constantly providing new challenges, such as on the periphery of the distribution of a species, greater genetic flexibility may be necessary for survival. This is borne out by the finding of a decreased number of inversions at the periphery of the distribution as compared with the center in certain populations of Drosophila (see later). Another mechanism for maintenance of a balance between stability and flexibility of the genotypic composition of the population is the alternation of sexual and asexual generations. In the asexual generation genotypes are kept stable. The change from asexual to sexual phase is related to change in environment. The sexual generation occurs when the environment becomes unfavorable and so provides the species at this stage with an increase in genetic variability (see Lewontin, 1957, for examples of this).

Huxley (1942) and more recently Lewontin (1957) have argued that there has been a general trend in evolution in which genotypic plasticity is gradually supplanted or replaced by phenotypic plasticity. Man's ability to alter his environment instead of being altered by it is an example of phenotypic plasticity, for this enables man to live in a wide variety of environments. Phenotypic plasticity does not necessarily involve the ability to alter the environment but more importantly the ability of a genotype to survive and reproduce in a wide variety of environments. Examples of this may be found more readily in the higher animals than in the lower. However it is not as yet possible to say to what extent one does supercede the other in passing from lower to higher organisms. Genotypic plasticity is, no doubt, the main method of adaptation in bacteria, protozoa and algae. It is a feature of many insects which have a high rate of increase (see Dobzhansky, 1951; Andre-

wartha and Birch, 1954; De Bach, 1958). Bullock (1955) has shown that plasticity of one sort or another is common amongst a variety of marine invertebrates but he makes no distinction between genetic and phenotypic plasticity in his review. Battaglia (1958) provided an example of genetic plasticity in a marine copepod and suggested a number of others. Nevertheless phenotypic adaptation to temperature alone in lower organisms has been demonstrated in bacteria, planaria, crayfish, crab, clam, limpet, various annelids and a great variety of insects (Fry, 1958). In the vertebrates genetic plasticity is well illustrated by races with different tolerances to temperature in the frog *Rana pipiens* (Moore, 1949) and in fish in the two-spined stickleback (Heuts, 1956). The stickleback also has races with different tolerances to salinity. The existence of genotypic plasticity in fishes is also evident from the success of fish culturists in selecting for higher fecundity and faster growth rates. On the other hand the great amount of work on acclimatization of fishes has been singularly unsuccessful in revealing much genetic plasticity. This may simply mean that genetic diversity is more difficult to detect in fish than in other animals. Some reasons why this may be so have been given by Fry (1957). Hart's (1952) study of freshwater fishes in the North American continent from widely different environments showed very little conclusive evidence for genetic diversity; on the other hand, his work and that of Fry (1957, 1958) shows that phenotypic plasticity in relation to temperature is common in freshwater fishes. Homoiothermy confers a high degree of phenotypic plasticity in birds and mammals making genetic plasticity less necessary in some circumstances. But of course genetic plasticity is still a feature of homoiotherms and without it evolution could not occur.

There is a succession of levels at which adaptation could be studied; the genes involved, the chemical intermediaries between the genes and the immediate phenotypic effects they produce, such as increased resistance to cold or increased ability to disperse, and the ultimate effect of these phenotypic changes on birth-rate and death-rate. The purpose of this paper is to relate genetics with birth-rate and death-rate, for it is birth-rate and death-rate which ultimately determine the number of animals. The immediate phenotypic expression of the genes is also the province of genetics and ecology but this will not be considered further here.

THE INFLUENCE OF GENETIC COMPOSITION ON THE RATE OF INCREASE "r"

Species differ in their capacity to increase in numbers during favorable periods when increase is possible. One species can be compared to another in this respect by comparing their innate capacities for increase or intrinsic rates of natural increase, r_m (Andrewartha and Birch, 1954, chapter 3). Such comparisons show quite clearly that species differ genetically in their capacities to increase in numbers. When we come to question how capacity for increase is selected in evolution it is necessary to discuss the rate of increase in terms of the actual rate of increase, r, characteristic of the natural environment in which the species lives. The statistic r_m is an abstraction

from nature in so far as it is a measure of a rate of increase when certain components of environment are excluded.

Natural selection will tend to maximize r for the environment in which the species lives, for any mutation or gene combination which increases the chance of genotypes possessing them contributing more individuals to the next generation (that is, of increasing r) will be selected over genotypes contributing fewer of their kind to successive generations. This is the usual meaning of fitness of a genotype. The tendency of natural selection to maximize r does not necessarily mean that natural selection will tend to make the numbers of the species a maximum. The maximization of r would tend to this end except in so far as mechanisms have evolved which put a limit to the numbers in unit space, irrespective of rates of increase. The extent to which such mechanisms may exist is discussed below.

The rate of increase is the difference between the two components of increase: birth-rate and death-rate. The maximization of r through selection does not necessarily imply that natural selection will tend to increase birth-rate or to decrease death-rate, but that it will tend to maximize the difference between them. An animal has a certain amount of energy to dissipate in its life. Some of this energy will go into functions associated with reproduction, some of it will go into activities such as dispersal and escape from predators and some of it will go into the functions associated with just being alive. The partitioning of energy between these various functions will be such as to maximize the chance for survival and multiplication. Selection for clutch size in birds and litter size in mammals are rather special examples as we shall see later. An increase in the size of the clutch or litter to numbers so great that offspring cannot get enough food for survival from the parents is unadaptive. There is good evidence that selection will tend to produce a clutch size corresponding to the number of young that can be successfully reared. This may vary from one part of a bird's range to another (Lack, 1956; Moreau, 1944). Similarly a balance has to be struck between the number of eggs laid by an animal such as a fish and the size of the egg. Survival of fry hatched from large eggs may be greater than survival of fry hatched from small eggs. Selection may favor a small number of large eggs rather than a greater number of small eggs (Svardson, 1949; Rodd, 1946). Tsetse flies have a birth-rate which is probably the lowest of any insect. The offspring are laid as mature larvae about to pupate and only one larva is produced at a time. Their energy is concentrated into a few offspring born in a mature stage of development rather than in a large number of eggs. Survival of the immature stages is made maximum by virtue of the advanced stage at which the young are born.

Evidence that natural selection has not pushed birth rates to the attainable maximum is provided by those domestic and laboratory animals in which artificial selection has been effective in increasing the birth rate or egg-laying rate. But this gain is purchased at the expense of other traits that would be important in survival in nature (see Cole, 1957; Smith, 1954).

In contrast to the tsetse fly, adult Mayflies (Ephemeroptera) produce a large number of eggs and then die after a brief adult life without feeding at all. There is presumably in these insects a selective advantage in concentrating the energy of life into early egg-production in the adult and survival of the long-lived immature stages, rather than in survival of the adult with egg production covering a longer period. An insect which can mature eggs without feeding, such as the Mayfly, has an evolutionary advantage when adult food is scarce over an adult which has to feed to mature its eggs. The adults of blowflies normally require protein in their adult diet for maturation of eggs. But Nicholson (1957) has produced a strain of the blowfly *Lucilia cuprina* which can mature some eggs without protein in its diet (from protein taken in the larval diet) by raising them in very crowded cultures where protein is in short supply. Insects which do not feed at all as adults have evidently taken this step some stages further.

These are a few of the variety of ways in which the life history of a species is patterned by selection and presumably in each case to maximize the difference between birth-rate and death-rate. Early production of litters, larger litters, closer spacing of litters and biassing of the sex-ratio in favor of females and higher survival of pre-reproductive and reproductive stages would each (other things remaining the same) increase r. Cole (1954, 1957) has pointed out that which particular ones which would be most effective depends upon the pattern of life history of the species. For example, species that reach maturity early can gain more from increasing litter size than from living longer and producing more litters. In species that mature more slowly it may be more advantageous to live longer and produce more litters than to increase litter size. Life history changes that involve a biassing of the sex-ratio are peculiar in the payment exacted from an evolutionary point of view. An increase in the proportion of females in the population may decrease the chance of females finding a mate. An answer to this may be found in asexual reproduction. But this would usually involve loss in genetic plasticity. A combination of the two may be best both from an ecological point of view (Cole, 1957) and from an evolutionary point of view (Wright, 1931). Whatever change occurs in the pattern of the life history a balance has to be found between the most appropriate reproductive and survival pattern.

Direct evidence of selection for rate of increase is provided by Bateman's (unpublished thesis, 1958) study of populations of the trypetid fruit fly *Dacus tryoni* collected from different geographic areas in Australia. He measured the innate capacity for increase r_m of population from four places along a 2,000 mile stretch of coastal country in Eastern Australia. In this example there is reason to suppose that the statistic r_m reflects the real capacity of the species to increase in numbers in the places where they live. There were significant differences in the capacities for increase of the four populations. These differences have probably developed through selection in the last fifty years, for evidence suggests that the fly has spread from its

tropical home into temperate latitudes during this period. The differences which he found were correlated with differences in temperature in the four places from which the populations came. For example, the most northern population (from Cairns in the tropics) had the lowest value of r_m at 20°C. and the highest at 30°C. The population from Sydney 1500 miles south had the highest value of r_m at 20°C. and the lowest at 30°C. Increase in the innate capacity for increase at 20°C. was doubtless one factor which enabled the species to become established in Sydney. The main evolutionary changes which occurred in the life history pattern to bring this about were increase in the number of progeny and in the life expectancy of the adult. For example, if we compare the populations from Cairns and Sydney we find that at 20°C. the number of progeny produced is greater for the Sydney population at all ages of the parent female and that they start laying eggs several weeks earlier. In the tenth week the Sydney strain lays nearly 30 times as many eggs as the Cairns strain. By the 20th week it lays about twice as many eggs. This may be an illustration of the principle that a rise in the innate capacity for increase can be most readily obtained by increasing the rate of egg production early in a female's life. The survivorship of adults of the Sydney strain is also greater at all ages than that of the Cairns strain at this temperature. The Sydney climate has evidently imposed a selection at 20°C. whereas in Cairns selection at such a temperature could hardly occur.

It would be a fascinating field of study to investigate the genetics of such evolutionary changes in life history patterns. A possible lead in this direction has been given by Carson's (1958) striking experiment with *Drosophila melanogaster*. He established experimental populations of flies over several generations in which the numbers fluctuated around a fairly constant level. He then introduced a "foreign" gene into these populations. There was a rapid three-fold increase in size of the population which was maintained for fifteen generations, after which the experiment was terminated. The increase in total numbers of the populations involves an increase in birth-rate or a decrease in death-rate or both. One gene was responsible for the change. His evidence was in favor of the hypothesis that these changes were the result of new heterotic combinations of genes produced after the foreign gene was introduced.

The evolution of heterosis following recombination and consequent change in fitness of genotypes has also been demonstrated in laboratory cultures of *Drosophila pseudoobscura* by Dobzhansky (1957). Seasonal changes in the frequency of chromosomal inversions in natural populations of this species in California (Wright and Dobzhansky, 1946) suggest that the rate of increase r of populations of *D. pseudoobscura* is a function of the different kinds of inversions in the third chromosome. Some combinations permit increase in the spring, others permit increase in the summer. In some unpublished experiments of my own I have found that when the trypetid fruit fly *Dacus tryoni tryoni* was bred together with the color variant known as *D. tryoni neobumeralis* in the one population cage, and provided that its initial fre-

quency was only 20 per cent, its rate of increase in the population declined until none were left after 35 weeks at 25°C. But when the initial frequency of *tryoni* was 80 per cent the rapid decline in numbers was halted after 20 weeks and this coincided with an increase in frequency of hybrids. From then on for the 100 weeks in which the experiment has continued *tryoni* has persisted in the population together with *neobumeralis* and their hybrids. The initial high frequency of *tryoni* gave this variant sufficient time to introduce its genes into the populations through the hybrids that were formed, so permitting a balanced population of the two types and their hybrids to evolve. Both *tryoni* and *neobumeralis* have changed genetically in this experiment, for the degree of sexual isolation that exists between the original populations had largely disappeared after 100 weeks. A genetic change in *tryoni* in this experiment has altered its rate of increase permitting it to persist in the mixed population whereas in the initial experiment its rate of increase decreased in the course of the experiment.

Family selection. The tendency of natural selection to maximize the rate of increase r poses a dilemma. It is easy to imagine that a low rate of increase could be advantageous under some circumstances in which large numbers would result in serious depletion of resources of food and space, with consequent starvation and death. The chance of annihilation from this cause would be reduced if the birth-rate were lowered and this resulted in greater chance of offspring surviving. However, selection for lower birth-rate can only occur when parents and progeny remain as a family during the rearing stages or in circumstances which are strictly analagous to a family situation. Selection for clutch size in birds is a classic example of selection operating on the individuals of a family unit. It is the families of optimum size that are selected. In social insects sterility for the bulk of the colony has evolved. This has happened because the colony is a family. Here it is the parents whose family organization is best for survival who are selected. Darwin recognized that sterile castes of social insects could only evolve as a result of selection operating on a family kept together as a unit. If the progeny of one colony mixed freely with the progeny from another then sterility could hardly evolve. This is sometimes called, with a lot of other unrelated things, selection in which the population is the unit of selection. This however is a misleading phrase for what is always selected are individuals. In selection for clutch size the individuals selected are those whose parents laid neither too many nor too few eggs. The regulation of the fecundity of the queen bee and the regulation of the number of reproductive individuals in the colony of the termite *Kalatermes flavicollis* are further examples of evolution of lowered birth-rate (Emerson, 1958).

Can selection favor the individual with a low birth-rate (other things being equal) in cases other than the closely knit family? The following hypothetical example is perhaps one sort of situation in which selection might operate in this direction. Let us suppose that a species of mosquito lays its eggs in pools of water with insufficient food for more than a few larvae. If each pool received eggs from one female only then the female which laid the

smaller number of eggs would tend to be selected. We shall suppose that those which laid large numbers of eggs produced no living progeny because of overcrowding in the pool. If different females laid their eggs together in the same pool then selection will not favor the mosquito which lays the smaller number of eggs. This would be analagous to mixing the clutches of several birds. However we could imagine an intermediate situation in which, despite the fact that mosquitoes laid their eggs in the same pools, some pools by chance might have eggs laid in them by mosquitoes of low fecundity. In so far as this occurred selection might favor the genotype of low fecundity especially if the smaller number of eggs is also correlated with larger eggs. In this model the pool is analagous to a family. I find some difficulty in supposing that this could be at all common in nature. The possibility should not however be overlooked. In these examples in which selection operates on individuals in a family, or something analagous to a family, selection still favors those genotypes with higher r, though in these cases the higher r is achieved by lowered birth-rate.

The general statement that selection will tend to maximize r is quite consistent with the fact that genotypes of low adaptive value may not be selected out of a population. In balanced polymorphism the homozygotes have lower adaptive values than the heterozygote. Similarly a genotype may have qualities of advantage to the species but of no advantage to itself. The theoretical concept of the "altruistic gene" (Haldane, 1932; Wright, 1949) is an example of this. Such a gene would increase in the population so long as the presence of such genotypes increased the chance of the population as a whole to survive and multiply. Too many of them might of course be disadvantageous. But even in this case selection still tends to maximize r for the population.

Territoriality. The tendency of selection is to bring r to a maximum. Yet there are numerous disadvantages in overcrowding and a high rate of increase would tend to produce overcrowding. The disadvantages of high numbers would be overcome if there were mechanisms to stop increase when density reached a certain critical maximum. Some forms of territoriality may serve this end. This does not involve selection for a low rate of increase but selection for cessation of increase when numbers are high. Such mechanisms would have the advantages which a high rate of increase confers without the disadvantage of high total numbers. Intraspecific strife in territorial muskrats may serve such an end. By fighting when their numbers reach a certain density in relation to cover muskrats prevent further increase in numbers (Errington, 1943). Muskrats only tolerate a certain number of their kind in any one marsh but when numbers are low they can increase at a fast rate and without serious intraspecific strife. Territoriality may in some cases serve to conserve resources, though there is no general agreement among ecologists that this is so. However, it does seem to result in lower numbers per unit area than would be the case without it. Territoriality is common in birds and mammals (Gibb, 1956; Hinde, 1956). But apart from ants (Brian, 1955) it seems to be rarely recorded in invertebrates with a few

possible exceptions such as dragon flies (Moore, 1952; Jacobs, 1955) and crabs (Crane, 1941).

The slaughter of drones in Apidae and Meliponidae has a similar effect of getting rid of excess individuals in the colony (Emerson, 1958). These are examples of special mechanisms which will tend to keep population density at a low level, possibly even at an optimum level. They are in a different category from the more common effects of increased density in reducing birth-rate and increasing death-rate such as is observed in crowded experimental cultures of beetles and blowflies. A characteristic of these cultures is the enormous number of insects per unit space despite the effect of high density in reducing r.

We may then have to think of natural selection as having two tendencies, on the one hand to increase the rate of increase (with its attendant advantages) and on the other toward the evolution of mechanisms which stop increase when a critical density is reached.

THE INFLUENCE OF THE NUMBER OF ANIMALS ON GENETICAL COMPOSITION

In laboratory populations of insects the birth-rate falls and the death-rate rises with increase in density. This has been demonstrated also in some natural populations of certain birds and mammals which exhibit territorial behavior (Andrewartha and Birch, 1954, chapter 9). It has often been assumed that selection will be greatest in crowded populations because mortality rate is greater. There are however selective differences between some genotypes which exist whether the individuals live in a crowd or not. Genotypes homozygous for lethal or semilethal genes are examples of this. Furthermore the selective differences between genotypes may be a function of density and instead of disappearing at low densities the selective values may even be reversed. In uncrowded populations of *Drosophila pseudoobscura* the inversion Chiracahua was favored over Standard. When larvae only or larvae and adults were crowded at a high density the Standard arrangement of the genes was favored over Chiracahua. When larvae and adults were crowded there was selective mortality of larvae but not of adults. When neither were crowded and there was no opportunity for selective mortality there were selective differences evidently in the rate of egg-laying (Birch, 1955). Similarly I have found a reversal of selective values in two color variants of the Queensland fruit fly *Dacus tryoni tryoni* and *D. tryoni neobumeralis* depending upon whether the adults and larvae were crowded or not. In a population cage at 25°C. the type *neobumeralis* was favored and increased in frequency when adults and larvae were crowded. In relatively uncrowded cages the type *tryoni* was favored over *neobumeralis* and increased in frequency.

Nicholson (1957) kept cultures of the blowfly *Lucilia cuprina* under crowded conditions supplying the population with a fixed amount of larval and adult food at regular intervals. In all ten of his cultures the character of the oscillations in numbers changed about the 400th day. Nicholson showed that this was due to a genetic change in the population. Acute

shortage of protein in the experiments had resulted in the selection of flies which could produce and lay eggs in complete absence of protein. Such flies would have a selective advantage in these experiments as there was a severe shortage of protein. Normal adults require protein for production of eggs. Presumably the flies which do not need it in adult food can mature eggs on the protein in their bodies which was derived from larval food. The shortage of protein was in this case caused by high density of flies in relation to the amount provided daily. Nicholson's experiments were not designed to tell if this peculiar quality of flies would be selected when flies were uncrowded but on a diet lacking protein.

Laboratory experiments with *Drosophila melanogaster* have demonstrated that selective differences in survival of larvae are a function of both the density of larvae and the genotype of larvae with which they are crowded. Lewontin (1955) found that a particular genotype was favored in the presence of certain genotypes but at a disadvantage in the presence of others. Parsons (1958) has since demonstrated the same thing.

The complexity of selection of a particular genotype in relation both to density and kind of genotypes with which it lives has been unravelled in a series of complex experiments with *Drosophila pseudoobscura* by Dobzhansky (1957), Levene, Dobzhansky and Pavlovsky (1954), Levene, Pavlovsky and Dobzhansky (1958). They have shown that the selective values of flies carrying a particular chromosomal inversion is a function of both the kind and number of other chromosomal inversions in the population. In population cages containing flies with different chromosomal inversions the selective values of the inversions change as the frequency of the different inversions change. The selective values of a particular inversion will depend not only on its own frequency but on the frequency and kind of the other inversions present in the population.

Oscillations. These considerations of laboratory experiments suggest that change in genetic composition in relation to density may provide a clue to the vexed problem of the causes of oscillations in numbers in certain animal populations. Oscillations are characteristic of confined populations of the beetles Tribolium and Calandra studied in the laboratory and also of some natural populations. Park has consistently found that when populations of *Tribolium confusum* are maintained in vials for long times with regular renewal of food the numbers undergo a long term oscillation. The time between successive peaks was about 500 days when cultures were kept at 29°C. Birch found that two species of Calandra grain weevils gave similar long term oscillations with about 280 days between successive peaks at 25°C. (see Andrewartha and Birch, 1954, chapter 9). These oscillations appear to be quite different in nature from the short term oscillations obtained by Nicholson with Lucilia which appear to be explained quite satisfactorily by the massive deaths of larvae or adults when food was completely exhausted. This never happened in Park's or Birch's experiments. It is conceivable that such oscillations as they found could be due to selection fa-

voring certain genotypes at high density and others at low density. As yet experiments have not been done to test this.

This hypothesis has been suggested as an explanation of outbreaks and declines of certain insect pests in Europe (Franz, 1950) and as a possible explanation of the unsolved problem of four year cycles in the vole *Microtus agrestis* in Wales (Chitty, 1957). Chitty has evidence of the existence of a hemolytic disease which is common when voles are dense and numbers are declining and rare when voles are increasing in numbers. There is some evidence that the disease may be genetic. Chitty postulates a gene or genes for the disease which confer some advantage on the voles living under crowded conditions at least upon heterozygotes and which is at a disadvantage when voles are not crowded.

When we come to examine the evidence from natural populations for the influence of density on selection and therefore on genetical composition little is to be found. This may simply mean that it has not been looked for. Williamson (1958) reviewed such evidence as is available and concluded from a number of suggestive cases that the only substantial one was Gershenson's (1945) work in Russia on change in proportion of black forms of the hamster *Cricetus cricetus* with change in number.

Drift. Fluctuations in numbers also suggests the possibility of genetic drift in isolated populations of small absolute size. Kerr and Wright (1954a, b, c) demonstrated the operation of random drift as well as selection in populations of very small size (four pairs). Dobzhansky and Pavlovsky (1957) have shown experimentally that the results of selection were different in populations of *Drosophila pseudoobscura* containing different chromosomal inversions, depending upon whether the initial population consisted of 20 or 4000 flies. Variability in chromosome frequency was very much greater between populations initiated with the smaller number of flies. Their explanation is that natural selection started with populations which were more like one another in the experiments initiated with many flies but with populations which were less like one another in the populations initiated with only 20 flies.

Extinction. A final aspect of small numbers is the problem of extinction. Because ecologists study species that are extant on the face of the earth today and not the much greater number of species that are extinct they are inclined to regard the existence of extinct species as a peculiar problem. They tend to pose the question thus—what prevents species from becoming extinct? In view of the fact that extinction seems to be the inevitable fate of all species it might be more realistic to put the question thus—what it is that enables species to remain extant for as long as they do in the face of changing environment? Ecologists have attempted to answer this type of question largely in the terms of ecology. It is claimed for example by some of the adherents of the so-called "density-dependent" school of thought that in the absence of "density-dependent" factors populations would quickly become extinct. An alternative point of view has been that the chance of extinction during a low phase in fluctuation in num-

bers is reduced by the patchiness of the environment or the discontinuity of the population in space. The genetic factor which may be all important in this discussion is the capacity of species to change genetically during unfavorable periods when its numbers are drastically reduced. Combinations of genes which will enhance the chance for survival and multiplication will tend to be selected. During such exigencies genetic plasticity is a safety factor reducing the chance of extinction. It is not of course a guarantee of the continuance of the species any more than either of the ecological arguments can guarantee survival of the species (Birch, 1957). In an environment subject to change, populations of the polymorphic species will become extinct less often than the monomorphic one.

The peculiar characteristics of diapause, hibernation and migration are adaptations which have presumably been evolved in relation to such adverse influences as extreme dryness and cold. Similarly peculiarities of behavior such as aggregation and the instincts which serve to bring the sexes together in sparse populations are evidently adaptations evolved to overcome the hazards of low numbers.

THE INFLUENCE OF OTHER SPECIES ON GENETICAL COMPOSITION

The genetical composition of a population may change in space and time as a result of the selective action of almost any component of environment (Andrewartha and Birch, 1954, chapter 15). Most of the examples in preceding sections were concerned with genetic change in relation to the numbers of the animal present and to weather. The numbers of another species present in an animal's environment may also be a selective agency. This has now been well established for a number of predators but as yet little direct evidence has been found of selection due to non-predators in an animal's environment.

The outstanding examples of the selective action of predators are Cain and Sheppard's work on predation by birds on the land snail *Cepaea nemoralis*, summarized by Sheppard (1958); the work of Ford and Kettlewell on industrial melanism in moths summarized by Kettlewell (1956, 1958, 1959); and Brower's (1958a, b, c) studies on selective predation on mimetic and non-mimetic butterflies. In each of these examples the authors have succeeded in demonstrating that predators tend to select the conspicuously colored prey so conferring an advantage on the cryptically colored individuals. Over 70 species of moths have evolved black populations in industrial areas in the last 100 years. Kettlewell (1959) has found a number of genetic mechanisms responsible for the dark forms. In some species a single dominant gene changes the animal from white to black in one step. In some populations of *Gonodontis bidentata* polygenes are responsible for darkness. In some populations of *Lymantria monacha* three dominant genes are responsible for producing the black forms. Kettlewell also quotes one case of blackness being due to a recessive gene and another where the gene was incompletely dominant.

Concerning the possible selective role of non-predators in the environment of an animal Brown and Wilson (1956) cite a number of examples of several species with overlapping ranges. They refer to various species of ants, frogs and birds in which the related species show more divergence in various characters in the region of overlap than elsewhere. Most of the variations they refer to are morphological but some are physiological. They suggest that the differences have been evolved in the region of overlap as a means of preventing gene flow between two related species. These may be examples of change in genetic composition as a result of the presence of another sort of animal, the change having taken place before the two forms were completely sexually isolated. However there is not any evidence on which to make a judgment as to whether this is simply a case of evolution of isolating mechanisms in the region of overlap or whether genetic change was promoted by selection in response to direct interference of one form by the other or in relation to shortage of common resource. Brown and Wilson assume the latter. It may have been either or both.

PERIPHERAL POPULATIONS

Populations on the periphery of the distribution present some special ecological and genetical problems. Usually the environment is more severe at the periphery. The ecological problem is how the species manages to survive there and secondly how it sometimes spreads from there to previously uncolonized regions. The occupancy of some peripheral areas is temporary or is reinforced by invasions from within the distribution. Thus the moth *Heliothis armigera* is found in places where it can not overwinter, such as in the state of Minnesota, as a result of migrations of adults from the south. At the periphery the species is usually rarer than elsewhere, a fact which Andrewartha and Birch (1954) interpret as being due to the shortness of the favorable period when numbers can increase before an unfavorable period and a negative rate of increase supervene. This is merely an intensification of factors which operate in a less extreme way elsewhere in the distribution. But the severity of the environment must impose extremely severe selection and this would seem to be corroborated by the findings of Townsend (1952) for *Drosophila willistoni* and Carson (1955) for *Drosophila robusta* that homozygosis, so far as chromosomal inversions are concerned, increases from the center of the distribution to the margins. In the marginal areas where environment is hostile few chromosomal inversions are successful. But those that are successful enable the species to survive there. Another aspect of this hypothesis is that a species with many chromosomal inversions can occupy a greater variety of environments than one with few. This concept is supported by the wide distribution of *Drosophila willistoni* and *D. paulistorum* which have 44 and 34 chromosomal inversions respectively and the very restricted distribution of its sibling species *D. tropicalis* and *D. equinoxialis* which have each only four chromosomal inversions (da Cunha et al., 1950; Dobzhansky et al., 1950). Chromosomal inversions con-

fer adaptability on the species in an environment which varies either in time or in space. Polymorphism in the center of the distribution is maintained at the expense of a certain degree of genetic plasticity which crossing over confers and which is presumably of greatest advantage at the margins of the distribution. There the species is on the frontier of its distribution and is subject to the constant threat of extinction. It is there that variability has to be welded into new combinations to meet a constantly changing and hostile environment. Speculation as to the importance of peripheral populations in evolution on a grand scale has been made by Mayr (1954) and Brown (1957) but these considerations go beyond the scope of this paper.

SUMMARY

The genetic plasticity of populations implies that the numbers of animals, which is what the population ecologist studies, may be a reflection of change in genetic composition either in space or in time. Alternatively the number of animals may itself be a cause of genetic change. These genetic aspects of ecology will be more important when genetic plasticity is more characteristic than phenotypic plasticity. In relation to changing environment genetic plasticity can only be adaptive when the length of the generation is less than the time the environment takes to change. Both types of plasticity exist throughout the animal and plant kingdoms. In some groups of animals in which phenotypic plasticity is common, as in fishes, it is extremely difficult to establish the extent to which genetic plasticity exists. Adaptation through genetic plasticity depends upon a continuous source of genetic variability. The source is mutation and in addition, in sexual organisms, recombination of genes. Sex also introduces the possibility of stored variability which is another source of variability available to the species. The sources of stored variability are, dominance, canalizing selection, linked polygenes in which crossing over occurs and heterosis. A balance between flexibility and stability of genetic composition is attained by a variety of genetic mechanisms of which chromosomal inversions and alternation of sexual and asexual generations are two.

From an ecological point of view the significance of change in genetical composition of a population is its effect on the rate of increase r, that is on birth-rate and death-rate. The tendency of natural selection is to maximize r. This does not mean that natural selection will tend to increase birth-rate to the absolute maximum possible and to reduce death-rate to the absolute minimum possible. But it will tend to maximize the difference between them. Life histories have been patterned by natural selection to this end. The rate of increase r can be increased by a variety of alterations to the life history pattern. The ones which will be useful will depend upon the environment the animal lives in. An increase in r may be achieved through natural selection by a decrease in the birth-rate. This can occur in one sort of situation only and that is when progeny and parents remain together as a family, at least during the rearing stage. This sort of selection has resulted in characteristic clutch sizes in birds and sterile castes in the social in-

sects. Selection for low birth-rate could, in theory, occur in non-family organisms under special circumstances in which the offspring of the one or a few parents tended to be reared in isolation from those of other parents. Although selection cannot favor a low rate of increase, it may favor the development of mechanisms which cause a cessation of increase when a certain critical density is reached. Some forms of territoriality may serve this end.

Concerning the influence of numbers of animals on their genetic composition, there is experimental evidence that some genotypes are favored in a crowd and others are favored when sparse. Further the survival value of a genotype is a function not only of the number of other genotypes around it but also of their kind. These findings in experimental populations may be important in resolving the causes of oscillations in numbers in some experimental and possibly in some natural populations; and secondly they suggest a role for genetic plasticity in reducing the chance of extinction.

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ON THE RELATIVE ABUNDANCE OF SPECIES

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This paper will contain a discussion of the ecological consequences which can be deduced from data on the comparative abundances of species found together.

Let $N_i(t)$ be the abundance of the i-th species at time t. Then if $r_i(t)$ is defined by

$$r_i(t) = \frac{1}{N_i(t)} \frac{dN_i(t)}{dt},$$

integrating, we obtain

$$\log N_i(t) = \log N_i(0) + \int_0^t r_i(t)dt. \tag{1}$$

Notice that r is permitted to vary. There are two opposed schools in ecology, one maintaining that the integral in equation (1) is important compared with $\log N_i(0)$ and the other maintaining that it is unimportant. These two views may lead to different ideas of the relative sizes of the $N_i(t)$ and so they will be discussed separately.

OPPORTUNISTIC SPECIES

Opinion 1: $\int_0^t r_i(t)dt$ *is important compared with log* $N_i(0)$. Such species are essentially opportunistic, being common when conditions have been good for some time; no equilibrium populations are maintained except perhaps as long-term averages. Basically, in this case, relative abundance is of little biological interest, because it is controlled by the vagaries of the climate and other aspects of the environment affecting r. However, it is often, but not always, possible to predict the relative abundance directly from equation (1). For, when the r_i vary completely independently, their accumulated integrals undergo "random walks" and will become normally distributed (Feller, 1950; Margalef, 1957, has already pointed this out.) And, by our assumption, the $\log N_i(t)$ will be normally distributed and the $N_i(t)$ will therefore be log-normally distributed. A difficult and perhaps more important case leading to a log-normal distribution is discussed later. Notice that this distribution reflects nothing about the structure of the community; common species are those which lately have had large r's; at different times, or different places (within the same habitat) different species will be the most abundant. These conclusions form the easiest way to recognize opportunistic species. Thus, diatom species in polluted rivers show a log-normal distribution of abundances, and have common species with large r and with ir-

regular distribution (Patrick, 1954, and personal communication). The insects studied by Ross (1957) had common species unpredictable in space and time; they too must be opportunistic. In fact, it is reasonable to suppose that many terrestrial invertebrates (Andrewartha and Birch, 1954) and plants (at least those characteristic of early stages in succession; Salisbury, 1942) fall in this category 1. And at least some vertebrates (for example, Cape May and bay-breasted warblers; MacArthur, 1958) are also opportunistic.

EQUILIBRIUM SPECIES

Opinion 2: $\int_0^t r_i(t)dt$ *is unimportant compared with log* $N_i(O)$. This is the case of species in some sort of population equilibrium. Here, the study of relative abundance is important and may reveal structural features of the whole community. In this case, there will be as many models of relative abundance as there are models of species population interactions, and the results require a more elaborate discussion. The total abundance of a species over its whole range is different from its density in a local habitat, and the relative abundances of species over a large area will thus be quite different from those in a small section of the area. Which will be easier to understand? Two features will often make the relative abundances over a small area easier to understand. First, the environmental changes are relatively great in a large area; no theory of relative abundance can be expected to predict these. Second, there is some evidence on the evolution of population size (Fisher, 1958, p. 112). The population unit which undergoes natural selection is the hypothetical deme, even though this can not be delimited sharply. Thus, if relative abundances are measured on areas of size comparable to that usually occupied by a deme, then we may expect an evolutionary law of relative abundance of the type qualitatively outlined by Fisher. Such a theory would be very interesting. Fisher suggests that common species will increase faster than rare ones. If, as a first approximation, we assume that the rate of fixation of beneficial genes is proportional to the population size, so that the improvement each year is about proportional to population size, then as shown by Cramer (1946, pp. 218–221), population sizes will become log-normally distributed. This conclusion is of interest since one of the prominent theories of relative abundance, due to Preston (1948), shows that this curve fits observed data fairly well. However for ecological purposes it is useful to try to avoid the genetic assumptions involved in an evolutionary theory, and thereby to make alternative hypotheses which have a more purely ecological meaning. There are two principal alternatives.

(a) *The total number of individuals of all species together is essentially constant,* so that increases in the populations of some must result in decreases in others. There is a fairly natural model for this alternative. It will be illustrated with an example. Suppose there are ten individuals and four species; represent the individuals by ten i's in a row. We can then

draw five vertical bars between the letters in any positions such that at least one bar is at each end of the row: ||ii |iiiii |iii |. There are now four spaces between bars; these represent the four species, and the number of i's in the spaces are the numbers of individuals in the species. In the example, the left-hand species has no individuals, that is, is not represented in the census, and the other species have two, five and three individuals. The essential characteristic of this model is that an increase in one species' abundance, that is, a change in the position of one of the vertical bars, automatically involves a corresponding decrease in the abundances of other species.

Here, and for the rest of this paper, it is assumed that the species whose abundances are being compared are of roughly equal size, so that an individual of one species is comparable to an individual of any of the others. It is thus reasonable to consider the relative abundances of the bird species feeding on defoliating insects, and, separately, to consider the relative abundances of the parasitic insects. It is, however, both unreasonable and uninteresting to consider the relative abundances of the combined populations.

(b) *The abundances of different species are truly independent.* Here, an increase in the abundance of one species has no necessary effect on that of any others. The appropriate model in this case is a sequence of i's with a pair of vertical bars determining the abundance of each species; the position of each pair of bars is independent of that of the pairs of bars which determined the abundances of the other species.

These alternatives do not yet unambiguously determine relative abundances. Relative abundances are precisely determined, however, if we can assume that the bars are placed randomly among the i's. Mathematically, this is equivalent to having all positions of the bars equally probable. Now it is well known that a given species is usually common in one or two of the many habitats present in a region. In others it will be rare or absent and in still others it will have intermediate abundance. It is also well known that the different species have different habitat choices. Thus a census taker by a change in choice of homogenous habitat is quite likely to have many species change radically in abundance. And thus, in any given habitat the abundances are likely to be quite random.

These models, combined with the random element due to the census-taker, should determine the relative abundance of species, of approximately the same sizes, in communities obeying one of the two alternatives, a or b.

In an earlier paper (MacArthur, 1957), the formula for the expected abundance of the r–th rarest species was shown to be, for alternative a,

$$\frac{m}{n} \sum_{i=1}^{r} \frac{1}{n-i+1} \tag{2}$$

where there are n species and m individuals, and for alternative b,

$$(3) \qquad \frac{\sqrt{n-r+1} - \sqrt{n-r}}{\sqrt{n}}$$

Alternative a (total number of individuals constant) was shown to be closer to the truth, for birds at least, than alternative b (species abundances determined independently). This suggests that the subdivided segments of the sequence of i's in alternative a should correspond to a useful biological property of the organisms. Hutchinson (1957) defined "niche" in very elegant terms and was able to show that his "niche" and the non-overlapping segments of alternative a are closely related. It is thus appropriate to refer to a as "niches non-overlapping" and b as "niches overlapping."

Before passing on to a more detailed discussion of the evidence bearing on these alternatives, a different way of describing the same alternatives will be mentioned. If instead of ranking the species according to abundance and calculating the expected abundance of the r'th rarest species as was done in equations 2 and 3, one calculates the expected number of species with a given abundance (that is, the number of species with one individual, the number of species with two individuals, . . , the number of species with i individuals, etc.) the following formulae result (see appendix for proofs):

For alternative a, non-overlapping niches, the most probable number g_i of species with i individuals is given by the solution of

$$(2a) \qquad \log_e g_i + 1/2g_i = \lambda i + \mu$$

where λ and μ are undetermined constants. This implies that if alternative a is holding, a graph of $\log_e g_i + 1/(2g_i)$ against i should yield a straight line. For alternative b, overlapping niches, the expected number g_i of species with i individuals is given by

$$(3a) \qquad g_i = a - bi$$

where a and b are constants. Thus a graph of g_i against i should yield a straight line. The formulations of the results of alternatives a and b given in equations 2a and 3a are the same as those usually given to studies of relative abundance, but to the present author it is easier to think in terms of the formulation of equations 2 and 3, which dealt with ranked species.

In figure 1, alternatives a and b are compared with a bird census (Saunders, 1936) covering all of Quaker Run Valley in New York; in figure 2, the censuses of the component habitats (pasture, orchards, mature oak-hickory, etc.) are plotted. The expected curves for the two alternatives, based on equations 2 and 3, are plotted for comparison in figure 1. It is immediately clear that while as expected the total population of the valley fits neither alternative, the small, more homogenous habitats plotted in figure 2 are in very close agreement with alternative a as expressed by equation 2. (Any curve parallel to that for alternative a in figure 1 shows agreement with alternative a). The general close agreement is also very evident from figure 3 which is compiled from large tracts of virgin areas (three in Mexico

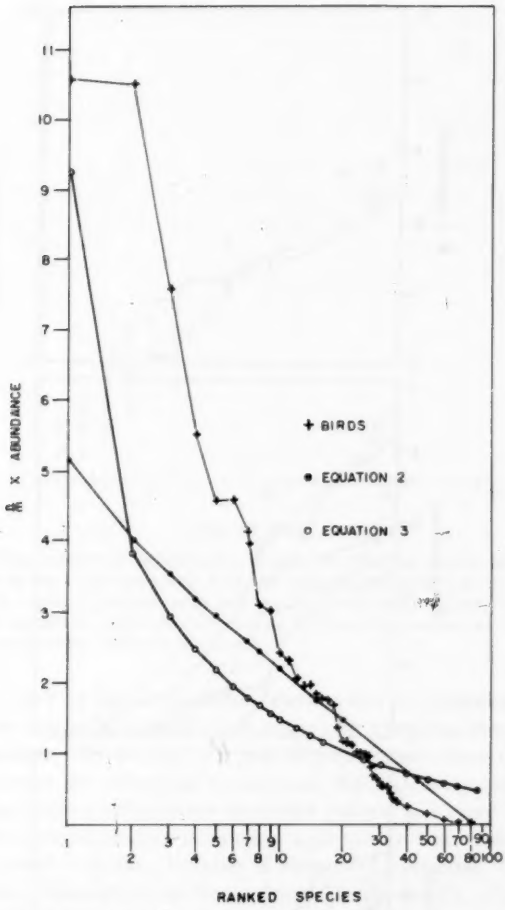

FIGURE 1. The abundances of the various bird species throughout Quaker Run Valley are ranked from commonest, on the left, to rarest, on the right, and plotted for comparison with the abundances expected from alternatives (a) and (b), which are given in equations 2 and 3.

[Davis, 1953, 1954, 1955], four in U.S.A. [Fables, 1957; Pugh, 1957; Longley, 1944; Hensley, 1954] and two in Canada [Stewart, 1955]).

These are all the censuses from virgin stands in extensive areas of that type which were analyzed. When bird censuses from mixed habitats are studied they reveal a divergence from the prediction of equation 2, however. This discrepancy is nearly always of the same type—common species are commoner than predicted and rare species are rarer—as was observed when the whole of Quaker Run Valley was compared with the formula of alternative a. Although two causes of this discrepancy will be discussed, the im-

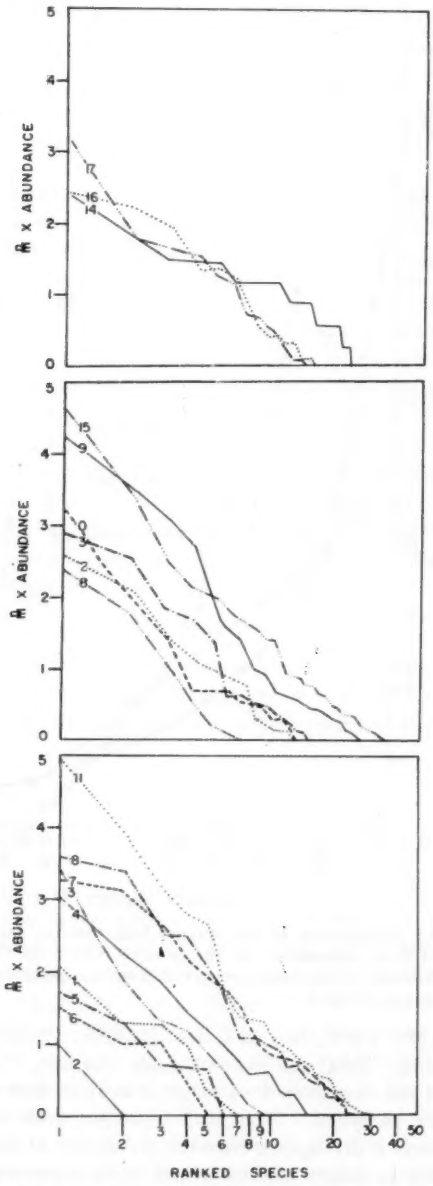

FIGURE 2. The bird species abundances of the component habitats of Quaker Run Valley are plotted separately, in the manner used in figure 1. A good fit to alternative (a) (equation 2) is indicated by the observed relative abundance curve being parallel to that curve in figure 1 which was calculated from equation 2.

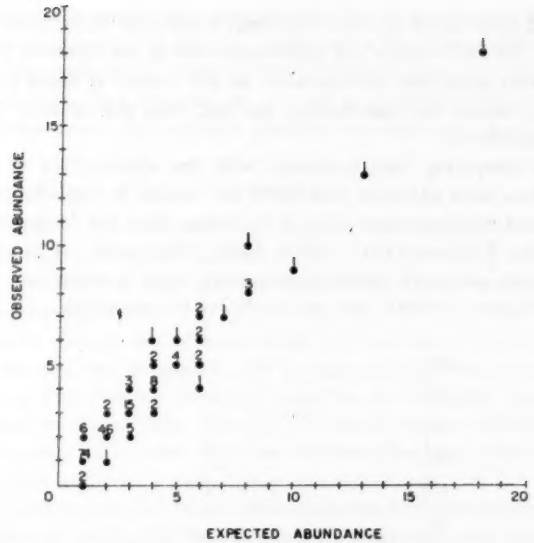

FIGURE 3. The observed abundances of species in nine tracts of virgin forest from Canada to Mexico are compared with the expected abundances calculated from equation 2. The expected abundances are taken as the nearest integer to the value predicted by the equation, and the parentheses enclose the number of species represented by the neighboring point on the graph.

portant thing to note is that it is always easier, that is, requires less information, to make a random system show excessive clumping than to make it excessively uniform. By moving any pair of points very close together one can always increase the clumping; to increase the uniformity one must have some knowledge of the configuration of nearby points, and move the pair accordingly. Thus any disturbance causing a departure from randomness is more likely to cause it in the direction of excessive clumping. Clumping in our model means clumping of the bars placed among the i's, which implies common species too common and rare too rare, as frequently observed. From a biological viewpoint, there seem to be two principal causes: (1) If the mean abundance of the species is different in two habitats, then the relative abundance in the combined area will depart from the expected. Thus, inclusion of an "edge" is likely to cause discrepancies. Hutchinson (1957) has appropriately called this phenomenon "heterogeneous diversity" and has discussed it thoroughly. (2) Empirically, common species in one habitat are more likely than rare ones to be common or at least present in neighboring habitats. For this reason, too, combining censuses from nearby habitats results in common species being unexpectedly common and rare species unexpectedly rare. Both of these causes operate in Quaker Run Valley where the individual habitats fitting equation 2 (and thus also 2a) combine to give a total census deviating greatly. Thus for birds at least it appears that the

postulates of alternative a—that the total number of individuals is roughly constant and the individuals are partitioned among the species present—are correct and that when the census-taker, by his choice of small homogeneous census area, makes the partitioning random, then the relative abundances conform to equation 2.

So far, in comparing bird censuses with the alternatives of equilibrium species, it has been assumed that birds are indeed in equilibrium. Perhaps the most direct evidence that this is so comes from the long-term censuses carried out by Williams (1947, 1948, 1949, 1950) over 18 years. These show that birds are more likely to decrease when common and to increase when rare. Figure 4 shows this strikingly for the ovenbird.

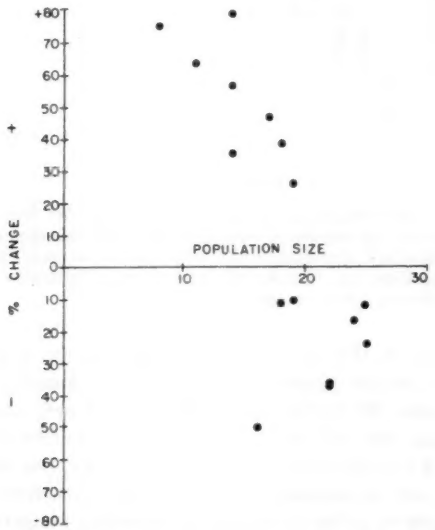

FIGURE 4. Increases and decreases over an 18-year period in the ovenbird population of a wood near Cleveland, Ohio, are plotted against the population size preceding the increase or decrease.

Data for other taxonomic groups are less convincing. Kohn (1959) has shown that Conus snails on Hawaiian reefs may agree closely with alternative a and equation 2, but otherwise, censuses of trees, fish, soil arthropods and other organisms deviate greatly from the expectation of alternative a and equation 2. Hairston and Byers (1954) feel that some species of soil arthropods show a local clumping and claim that this is not due to environmental heterogeneity. In this case, the environment is not being fully utilized and the postulates of alternative a, of course, fail to hold. Any closer agreement obtained by combining censuses from different areas is thus probably spurious. In the case of the trees, fish and perhaps some soil arthropods too, the census-taker almost certainly fails to effect the random subdivision

of the individuals among the species, so that even if alternative a still holds for these groups, the relative abundances from censuses would not be expected to fit equation 2. Many of the censused areas are probably heterogeneously diverse also, so that an attempt to deduce ecological properties of these groups from their relative abundances cannot be made along these lines.

SUMMARY

1. A distinction is made between opportunistic and equilibrium species.
2. There is little ecological interest in the relative abundances of opportunistic species, but such species abundances should frequently have a log-normal distribution.
3. The relative abundances of equilibrium species are of considerable ecological interest and frequently can be deduced from the assumption that increase in one species population results in a roughly equal decrease in the populations of other species. To make the formulae well-defined it is necessary to assume that the census-taker has sampled a small area and thus achieved a certain sort of randomness.
4. For bird populations, at least, discrepancies between observations and predictions are negligible except when the censused area is compounded from different habitats. The discrepancy is then partly due to the fact that common species in one habitat are more likely to be present in adjacent habitats than are rare ones.

ACKNOWLEDGMENTS

G. E. Hutchinson has provided a continuous stream of good ideas on the subject for seven years since he first drew the author's attention to it. Drs. Peter Klopfer, Monte Lloyd, and David Lack made valuable comments and criticisms. The work was supported in part by a regular postdoctoral fellowship of the National Science Foundation which the author spent at the Edward Grey Institute of Field Ornithology at Oxford, England.

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APPENDIX

Suppose there are n species and m individuals. Let g_i be the number of species with i individuals. The *most probable* values of g_i will be computed for each type of randomness and for alternative a and b. To simplify manipulations it is useful to remember that, by Stirling's formula (cf. Feller, 1950, p. 43) $\log_e n!$ is very closely approximated by $\log_e (2)^{1/2} + (n + 1/2)\log_e n - n$, and thus the derivative of $\log_e n!$ is very nearly

$$\frac{d}{dn} \log_e n! = \log_e n + \frac{1}{2n}. \tag{4}$$

Consider now the various cases. First, suppose total abundance is constant (niches non-overlapping) and the bars dividing the i's are moved randomly (niches continuous). As pointed out earlier, this means the i's are indistinguishable, and distinguishable combinations of i's and bars are equally probable. The number of distinguishable arrangements producing g_1 species of abundance 1, g_2 with two individuals, . . . g_i with i individuals, . . . , is

$$\frac{n!}{g_1!\, g_2! \cdots g_i \cdots},$$

and since there are, in all, n species and m individuals,

$$\sum_i i g_i = m, \quad \text{and} \quad \sum_i g_i = n. \tag{4'}$$

By Lagrange's method of undetermined multipliers the state of greatest probability will be given by the solution for $g_i (8 = 1, 2, \ldots .)$

$$\frac{\partial}{\partial g_i}\left(\log_e \frac{n!}{g_1!\, g_2! \cdots g_i! \cdots} + \lambda \sum_i i g_i + \mu \sum_i g_i\right) = 0; \tag{5}$$

In view of 2, this is equivalent to

$$\log_e g_i + \frac{1}{2g_i} = \lambda i + \mu. \tag{6}$$

It would be possible to solve for λ (approximately $-m/n$) and for μ (approximately $\log (n^2/m)$ because of conditions 4′, but this seems unnecessary since equation 6 implies that a graph of $\log_e g_i + \frac{1}{2g_i}$ against i will be a straight line, provided the hypothesis (continuous, non-overlapping niches) is valid.

Consider, next, alternative b. Here the species abundances are determined independently, or equivalently, two bars are placed randomly among

the i's and the number lying between them represents the abundance of a species; the process is repeated for each species. The total number of i's in the sequence is clearly not equal to the total number of individuals in the census, this time; it is an independent parameter, but fortunately one which has little effect on the relative abundances predicted. The problem is most easily solved as follows. Suppose, for instance, that there are ten i's. Construct a square array of 100 i's.

i i i i i i+i i i i
i i i i i+i i i i i
i i i i+i i i i i i
i i i+i i i i i i i
i i+i i i i i i i i+
i+i i i i i i i i+i
+i i i i i i i i+i i
i i i i i i i+i i i
i i i i i i+i i i i
i i i i i+i i i i i
+

Now two bars determined the abundance of the species in question; draw a vertical bar in the array in the position of the first bar determining the abundance, and a horizontal bar through the array in the position of the second bar determining abundance. Since each bar falls with uniform probability in each of the 11 positions available to it, all $11^2 = 121$ positions of the point of intersection of the two bars are equally probable. The probability that the two bars in the original sequence or the two coordinates in the square array differ by precisely four i's is then the fraction of the 121 coordinate points which satisfy this condition. The coordinates marked with a + sign are the ones, and their number is clearly $2(11 - 4) = 14$. In general, if there are p i's, the probability that two randomly placed bars enclose q i's is clearly given by

$$\frac{2(p + 1 - q)}{(p + 1)^2}.$$

When this probability is plotted against q, a straight line results. Therefore, since the expected number of species with q individuals under this alternative is proportional to the probability of q individuals, the number of species with q individuals would, if alternative b holds, be linearly related to q. Notice that the state of maximum probability was computed for alternative a while the mean or expected state was computed for alternative b. Presumably both distributions are sufficiently symmetrical so that the mean is approximately the state of maximum probability and conversely.

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CELL CONTACTS: SOME PHYSICAL CONSIDERATIONS

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Recent reviews of the behavior of cells (Abercrombie, 1958; P. Weiss, 1958) have mentioned the importance of reactions which depend on their contact. Many of these reactions, such as contact guidance (P. Weiss, 1934; P. Weiss and Garber, 1952), contact inhibition (Abercrombie and Heaysman, 1953) and the sorting out of cell types in reaggregation (Galtsoff, 1925 a,b; Lehn, 1953; Moscona and Moscona, 1952; Townes and Hollfreter, 1955) are expressed in the alteration of movement or adhesiveness of the cells. Abercrombie (1957 b, 1958) and Ambrose (1958) have stressed the importance of the nature of cell contacts in the malignant properties of cancer cells. The locomotory and adhesive reactions as well as others are at present not fully explained in terms of physical or chemical mechanisms. Schmitt (1941), Steinberg (1958), Tyler (1947) and P. Weiss (1941, 1947, 1953) have proposed that the cell types have various specific surface constitutions, such that whether two cells adhere or separate is decided by the similarity or dissimilarity of their surfaces. But these schemes are not easily amenable to experimental investigation, and moreover it has not been explained in detail how such similarities or the converse are translated physically into the phenomena of adhesion or locomotion. In this paper a different approach is proposed. Some of the physical properties of surface layers will be described, in particular those connected with the movement of surfaces and with their adhesion. From this basis explanation of several sorts of contact phenomena will be made.

SURFACE RHEOLOGY OF CELLS

A widely accepted model for cell-surface structure is that proposed by Danielli and Davson (1935), in which a bimolecular layer of lipid is covered with globular protein on either side. Robertson (1959) has added evidence from electron microscopy to support this scheme. These protein layers are thought to be monomolecular. In the following discussion the model that Danielli and Davson proposed will be used, but it should be realized that the phenomena which such a model may show may also appear in cell surfaces of different structure.

The protein layer on the outside of cells (when it is present) or any other outermost layer will tend to behave like a monolayer, and the discussion will be developed in terms of monolayers. Now it may not be a true monolayer, and the rheological properties of the cell surface may be partly those of the bulk phase inside it. Joly (1954 b) and Tschoegl (1958) have shown that the rheological properties of a monolayer may be directly transformed into those for a bulk phase. The rheological behavior of monolayers may not

apply strictly to cell surfaces, but if it does not the bulk phase equivalents will (particularly those applying to boundary layer conditions). Policard (1926) and Harvey and Danielli (1936) have already considered cell behavior in terms of the properties of thin films. Although the electrical and contact properties of the monolayer at the cell surface may be influenced by the orientated structure of the lipid beneath, the actual contact properties of the cell will be chiefly expressed by this outermost layer. For these reasons it is of interest to consider how surface rheology may apply to the behavior of cells.

Joly (1948, 1949) showed that protein monolayers behave as newtonian liquids when the surface area per molecule is large. If the surface area is reduced, their flow relations become non-newtonian at a certain point. Their viscosity then becomes a function of the shear applied. At surface pressures just above that at which non-newtonian behavior begins the effect of shear is to reduce the surface viscosity, but at higher pressures shear acts to increase the viscosity. At still higher pressures the surface layer gels. Similar phenomena were described by Inokuchi (1953). The equivalent phenomena in bulk phases are respectively thixotropy and rheopexy (Joly, 1954b).

Joly (1946a, b; 1950a, b; 1956) has provided an elegant explanation of these phenomena of surface thixotropy and rheopexy. Joly (1948) has shown that in protein monolayers the effective elements of the surface are not whole molecules but only portions of them, and the term "area per element" instead of "area per molecule" will be used. If such a monolayer is subjected to shear, a perturbation of the arrangement of the elements of the surface will result. When the relaxation time of the perturbation is comparatively large the shear will induce a second perturbation before the original perturbation has vanished. The activation energy of this second perturbation will be smaller than that of the first as there is less deformation of the surface structure (Joly, 1950a, b, 1956). As a consequence of the deformation of the surface structure a pressure appears, which tends to lead to expansion of the surface. Because of the breakdown of the surface structure under shear, the viscosity coefficient decreases.

Conversely, the surface viscosity rises if the shearing force promotes the formation of a sufficient number of more condensed packings as the elements of the surface pass one another during its flow. The probability of this aggregation depends on the shear and will also be more pronounced in closely packed surfaces than in surfaces of greater area per element. Moreover, the appearance of closer packings is in fact a reduction of the surface area per element, and is accompanied by a rise in surface viscosity; this rise leads to surface rheopexy.

It is of interest to see how the viscosity increase or decrease under shear is related to the surface area per element. The following results can be deduced from the papers by Joly (1946a, 1954a, b, 1956). If the molecular areas are large, it is unlikely that groupings will come into sufficiently close contact for more compact surface arrangements to develop; likewise,

movement of an element will have little effect on neighboring ones, so that the viscosity decrease under shear will be small. If the surface is very condensed, a change producing still further condensation will have a very high free energy of activation and there will also be a great resistance to the movement of one element independently of another. As a result, viscosity changes with shear will be strongly opposed. At intermediate surface areas, surface thixotropy or rheopexy will develop. There will of course be an intermediate surface area value at which the thixotropic effect is balanced by the rheopectic effect, and at which shear has no effect on viscosity. So if surface area (at rest) is plotted against viscosity increase or decrease under shear, the sine wave type of relation is obtained which is shown in figure 1.

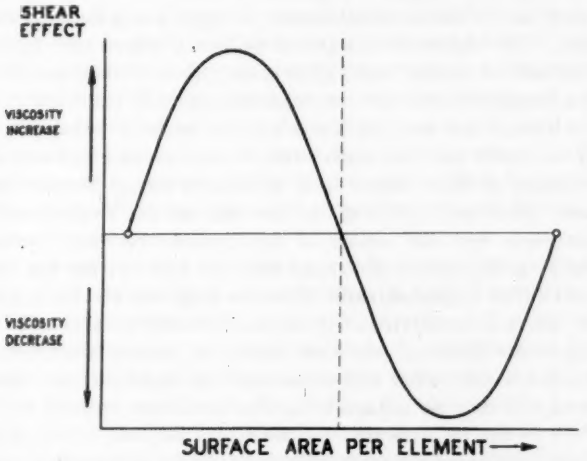

FIGURE 1. The relation between the non-newtonian viscosity and the area of the elements of the surface is shown in diagram. See also figure 2.

The relevance of non-newtonian surface viscosity to cell behavior is that the cell surface is subject to shear whenever it moves. Thus cell movement will induce viscosity changes in the surface, which will act back upon the movement itself, because the rate of "flow" of a surface and its undulatory behavior are affected by its viscosity (Rouse, 1950). However, does the protein layer on cell surfaces exist in the right condition to show such behavior? Harvey and Danielli (1938) pointed out that the protein on the cell surface was probably in the globular condition. The results of Inokuchi (1953) suggest that it is undenatured globular protein which shows this form of flow behavior in monolayers. There is reason (Joly, 1948, 1949) for thinking that surface layers of a wide range of proteins show very similar rheological behavior. The visco-elastic properties of the cell surface (Harvey and Danielli, 1936, 1938), moreover, are an expression of the non-newtonian

flow, for the relaxation of the surface structure will produce these properties. Thus it seems likely that the cell surface is in a condition to show non-newtonian viscosity. Such a condition would agree with surface tension measurements, which show very low values for the tension (Harvey, 1936), for low surface tension values imply high surface pressure values, and the results of Joly (1948), Inokuchi (1953) and Hamaguchi and Isemura (1955) show that non-newtonian viscosity does not appear until a fairly large surface pressure has been reached.

MECHANISMS OF ADHESION

Derjaguin (1954) and Verwey and Overbeek (1948) have described the theory of the interaction of two surfaces; it has considerable support from experiment. Surfaces attract one another with their van der Waals-London forces if they are of similar constitution, or repel one another if they differ considerably. The electrical charges of the two surfaces tend to drive them apart if they are of similar sign, or to attract them if they are of opposite sign. The electrical forces decline exponentially with the distance from the surface. Although the van der Waals-London forces between two isolated atoms vary inversely with the sixth power of the distance between them, the distance relation of these forces is of a different nature between two parallel surfaces. Overbeek (1952) shows that the van der Waals-London forces decline inversely with the square of the distance between two surfaces. Consequently, at increasing distances from the surface, the van der Waals-London forces tend to predominate. However large the electrical forces are, the van der Waals-London forces will exceed them beyond a certain distance. If the surfaces are identical (the case treated by Verwey and Overbeek), the van der Waals-London forces will attract and the electrical ones repel. The two surfaces will thus be attracted together until they come to the distance at which the forces of attraction and repulsion balance. This will be the point of stable adhesion. Van den Tempel (1958) has calculated and measured this distance for an oil-water-oil system with an electrolyte concentration of 0.01M to 0.2M sodium salt. He found that a stable adhesion is formed when the surfaces are 100 to 200 Å apart. Verwey and Overbeek point out that at 5-15 Å from the surface the van der Waals-London forces go to large values so that attraction again predominates in this region, though from 0 to 5 Å there is another region of repulsion. Between these two regions of attraction there is a region of repulsion, from c 15-100 Å where the repulsive force is in most cases so large that the energy of Brownian motion of the particles will be insufficient to surmount it. In consequence the surfaces of the particles cannot be brought into the inner region of attraction (Van den Tempel, 1958). Reduction of the electrical charge on the surfaces will bring the point of balance of the attractive and repulsive forces closer to the surfaces. As a result the van der Waals-London forces outside this point will be larger, and thus adhesions will become closer and more firmly held against breaking. Overbeek (1952) has calculated the interaction of repulsive and attractive forces for a large number of different cases. A

significant result of these calculations is that the potential barrier due to electrical forces remains large except for extreme cases. Consequently the close adhesions of the 5 to 15 Å range seem improbable for like surfaces.

Schmitt (1941), Townes and Holtfreter (1955), Tyler (1946) and P. Weiss (1941, 1947, 1953, 1958) have proposed schemes to explain selective adhesion. These theories maintain that adhesion is due to the close linking of the chemical groups of each surface with one another or with an intervening cement. The types of bonding proposed are covalent, co-ordinate, polar or hydrogen bonding, which have a range of a few angstroms. The specificity of adhesion is explained through the "keying" in of a pattern of binding groups on one surface with those on another, and is supposed to resemble the antigen-antibody interaction. According to these schemes the distance between the two surfaces will be a few angstroms. This does not agree with the conclusions of the Verwey-Overbeek theory, which applies to like surfaces. Large areas of the surfaces of like cells are alike in overall properties, but in details contain complementary patterns of the groups so that they can "key in." If these patterns are built up in charged groups, the theory of the adhesion of unlike surfaces may be applied. Derjaguin (1954) and Bierman (1955) point out that surfaces unlike in respect to the sign or density of charge can attract one another to give a final separation of 5 to 10 Å. The more unlike the electrical properties of each surface, the more easily does the adhesion form.

Now the interaction of two surfaces will become appreciable when they are separated by several hundred angstroms. At this distance the forces of electrical repulsion at any given point will be a summation of the negative and positive charges over the whole surface. The overlapping of the electrical fields caused by each charge becomes more pronounced the greater the distance from the surface; in other words, the electrical force pattern becomes more obscured. Obviously the interaction of patterned surfaces at a distance will be of the Verwey-Overbeek type, that is, they will behave as identical surfaces. If this interaction allows them to attract one another until they are close enough for the local differences in the electrical forces due to the pattern to act, then a close adhesion may develop, in the manner described for unlike surfaces. The larger the dimensions of the pattern—in other words, the greater the size of areas of one charge sign—the further away from the surface will appreciable patterning of the electrical forces extend. On this basis the probability may be assessed of surfaces having sufficiently large patterns for the conversion of the interaction from that for like surfaces to that for unlike surfaces. If the patterning is inappreciable at the distance at which the general electrical forces come to balance with van der Waals-London forces, then the close type of adhesion will fail to develop.

On topological grounds, if all the cells of one type are to have similar surface properties, the areas of positive or negative charge concerned in the pattern responsible for specific adhesion on each cell must equal each other and be the same as those on other cells. Moreover, the charge densi-

ties in each type of area must be equal. If these were the only charges, the surface would be at its isoelectric point. But under physiological conditions most if not all cells have a negative charge, so we must assume that there are a certain number of charges on the surface unconnected with the pattern. On this basis a calculation can be made of the minimum size of a region of one type of charge which would have an appreciable effect (that is, greater than five per cent) on the repulsive forces at 100 Å separation of the surfaces. This separation is chosen because it is the closest approach likely for surfaces on the Verwey-Overbeek theory, and is the closest usual separation of cell membranes (Robertson, 1959). On the topological grounds given above such an area must be met on either side by areas of opposite sign but equal dimensions. If the regions are considered to be long strips, their minimum width would be 25 Å. If they were square the dimensions of each side would be greater than this value.

Cumper and Alexander (1950) found that the amino acid residues of proteins in monolayers have an area of 14 – 16 $Å^2$ at the point where surface viscosity becomes appreciable. In consequence the minimum width of an effective strip pattern of protein would contain six amino acid residues in its width. That each amino acid residue in the region should have the same ionization and that there should be no charges of opposite sign seems most improbable. Davies (1954) has shown that the charged groups of each amino acid in a protein monolayer at an oil-water interface will protrude into the aqueous phase. On these grounds there would be very little possible patterning of charges, for at or near the isoelectric point there are many groups of either sign of charge in a protein. For these reasons it seems unlikely that the theories of adhesion which depend on the keying in of a pattern of charges explain the adhesion of like cells.

A somewhat different scheme to explain the specificity of cell adhesion has been proposed by Schmitt (1941) and Steinberg (1958). The cell surface is presumed to carry an orderly arrangement of groups capable of binding calcium. Like cells have like patterns on them. If two like surfaces come in contact, calcium ions cross-link the surfaces; if unlike surfaces come into contact, very few of these cross-links will develop. This type of theory runs into the trouble that the similarity of charge of the two surfaces will prevent close interaction, for the Verwey-Overbeek theory predicts a separation of 100 Å to 200 Å. Moreover, as Hill (1956) has shown, the shifting of calcium atoms from a linking to one surface to a binding link will not happen until the surfaces are closer than some 10 Å.

Thus it seems that we are driven back to reconsidering the explanation of cell adhesion on the Verwey-Overbeek model. The 100-200 Å separation between adhering surfaces predicted by this theory receives remarkable support from electron micrographs of cell contacts, which show a separation of this range of values (Mercer, 1957; Robertson, 1959). It might be argued that this separation is an artefact due to surfaces which were formerly patterned being made alike by the process of fixation, and in consequence altering the distance of separation to the larger value. Since like surfaces will be in a

region of attraction if they happen to be 5–15 Å apart it seems unlikely that fixation could have this effect. An attempt to explain selective adhesion on the Verwey-Overbeek model will be made later in this paper.

The application of the theory of Verwey and Overbeek to cell behavior allows us to make certain propositions that can be or have been experimentally tested. Highly charged surfaces should be less adhesive than those of less charge: this was found to be the case by Ambrose, James and Lowick (1956) and Ambrose (1958). The extreme importance of calcium in cell adhesion (Herbst, 1900; Schmitt, 1941, and Steinberg, 1958) has usually been explained in terms of its action in stabilizing a cementing material between the cells (Hober, 1945). However, calcium will have a very considerable effect in reducing the surface charge of protein and other charged surfaces. Bungenberg de Jong (1949) points out that the negative charges on colloids are reduced much more effectively by divalent than univalent cations. The same charge reduction is produced by a given calcium concentration as by a concentration of sodium ten to a hundred times greater. Thus it can be seen that calcium would aid cell adhesion by reducing the surface charge so that the repulsive force would be lessened. In consequence, the forces of attraction would result in a closer and firmer adhesion.

In this theory of cell adhesion the presence of a cementing material between the cells is not necessarily required. Since there will be a gap of 100 to 200 Å between the cell surfaces, this gap will contain intercellular fluid and such fragments of organic matter as may have become adsorbed on the surface. In consequence, electron micrographs will show only slight evidence of material in the intercellular space, and this is so (Mercer, 1957). However, the theory does not in any way deny the later development of cementing materials between cells in prolonged contact.

DYNAMIC AND STATIC ASPECTS OF ADHESION

It was mentioned above that reduction of surface charge would strengthen adhesion on the Verwey-Overbeek scheme. The density of surface charge can be altered by expanding or contracting the surface (Adam and Harding, 1932; Davies, 1951 a, b; Klevens and Davies, 1957). The van der Waals-London forces are hardly affected by this. Thus any effect which tends to expand the cell surface will tend to increase adhesion. However, this may be complicated by the unfolding of surface elements so that more charged groups are exposed, but this effect cannot be quantitatively assessed at present. In the theoretical picture of surface viscosity which was described above, the viscosity effects are dependent on the area of the elements of the surface. This will also be true of the adhesive properties. When the surface is at rest it will have a certain adhesiveness. If there is a viscosity decrease under shear, this will be accompanied by a surface expansion and a consequent rise in adhesiveness. A viscosity increase under shear will run parallel with a surface contraction and a fall in adhesiveness. If the surface viscosity decrease leads to the formation of an adhesion, this will tend to oppose the motion of the surface. As a result the shearing force will fall

and in turn a relaxation of the surface will tend to occur. Since the perturbation of the surface which has led to the viscosity decrease has an appreciable relaxation time constant (or else there would be no viscosity decrease) the adhesion will remain for a while after the shear has been removed.

A striking example of these phenomena may occur in the motion of the undulating membrane of the fibroblast. Ambrose and Curtis (unpublished, 1959), using the surface contact microscope, have noticed that cell contacts, which appear to be adhesive with the substrate, are formed by the "crests" of the undulations alone. The interpretation is that as the cell moves on the surface the regions of contact undergo shear. Consequently their adhesiveness increases and where this happens an adhesion will form. The adhesion prevents motion in that part of the membrane so that an adjacent region takes over the motion. After a time relaxation occurs and the adhesion breaks, but meanwhile another region has become adhesive. Consequently the adhesive region moves along the cell slightly behind the actively moving part.

In figure 2 the various relations between shear and surface area and adhesiveness are shown in diagram. The adhesive increase or decrease will alter the adhesiveness shown under no shear (static adhesiveness) to a new value, the dynamic adhesiveness. The initial surface viscosity is that at zero time of shear. If adhesion occurs the shear is destroyed, and after a relaxation time the increased or decreased adhesiveness vanishes.

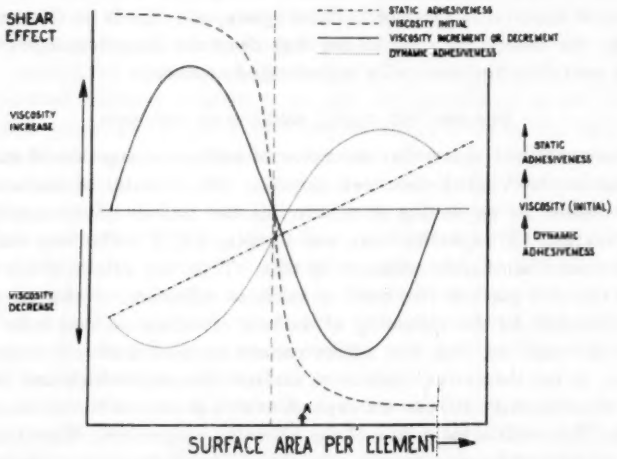

FIGURE 2. Here are shown the relations between the surface viscous properties and the adhesiveness which cells may have. The two curves relating to viscosity are confirmed by experiment on artificial surfaces (Inokuchi, 1953, Joly, 1956). The curve for viscosity increment or decrement on shear is that shown in figure 1. The letter A indicates the supposed surface nature of a reaggregating cell which is just about to start moving amongst other cells in a reaggregate (see section on reaggregation).

Fibroblast behavior in tissue culture. The example from the movement of fibroblasts will be developed. The main features of fibroblast behavior which it will be necessary to explain are the movement and form of cells and relations with other cells. The undulations of the fibroblast surface spread backwards along the cell from its leading edge. This leading edge is a widely spread fan-shaped extension of the cell, when it is on a flat surface (Abercrombie and Ambrose, 1958). The undulations move away from the leading edge and along the rest of the surface in the direction of the hind end of the fibroblast. However, there is some slight undulation of the surface that appears at the side of the cell and moves a short distance inwards. These waves lessen in amplitude towards the center and hind end of the cell, save that on occasion there is some beating inwards at the hind end. A dense monolayered outgrowth develops from the explant in which the cells lie packed close side to side and head to tail. There is a radial orientation of the fibroblasts with respect to the explant. They tend to move in such a way that the outgrowth is maintained as a coherent sheet, though at the periphery there are large open spaces laterally between the cells.

Many of these features are explainable by assuming that the fibroblast surface shows appreciable viscosity reduction under shear; and this will correspond with a fairly high static adhesiveness and a very high dynamic adhesiveness (see figure 2). It was pointed out that in such a surface as this, motion tends to be opposed by adhesion. The lower the viscosity of a medium, the more easily will waves form at its surface (Rouse, 1950), and the properties of the surface affect wave motion considerably. It should be noted that the visco-elastic properties of the layers immediately beneath the surface are of importance in cell movement, but this is beyond the scope of this paper. Thus the viscosity reduction under shear of the surface will aid wave formation but will also promote adhesion. Without some adhesion it would be impossible for the cell to get sufficient purchase on the surface for movement to occur. The interplay of these factors will set a limiting speed for the movement of a cell, other matters such as energy supply being equal. Those types of cell which are very adhesive will have great difficulty in moving at all, likewise those cells which have very little adhesion will be immobile (unless they take to swimming). Between these extremes there will be cell types that can show appreciable movement over a surface but which will also display some degree of adhesion.

The leading edge of a fibroblast or other type of cell is the first part to emerge from an explant, and in consequence it will be the first part of the cell to undergo shear against the substrate. This shear will lead to an increased adhesion and a surface expansion, which together may be responsible for the fan-shaped, spread-out form of this pseudopod. P. Weiss and Garber (1952) and P. Weiss (1958) have pointed out that it is unusual for an isolated fibroblast to bear more than one large persistent pseudopod, and moreover other smaller pseudopods are very rare. Abercrombie and Ambrose (1958) and P. Weiss (1958) suggest that if this leading pseudopod vanishes for some reason, then and only then does a new leading edge appear in some

other part of the cell (thus moving it off in a new direction). In other words, it appears that a leading edge has some kind of dominance that prevents the appearance of competing pseudopodia. This can be explained as a consequence of the surface expansion caused by shear in the leading membrane. The expansion will tend to lead to a contraction in the rest of the fibroblast surface, and this in turn will diminish adhesiveness and cause an increased surface viscosity. These factors will act against motion of the surface and against the effects of viscosity reduction under shear. Therefore there will be little chance of a leading pseudopod developing in the region outside the existing one. If the existing leading edge disappears the remainder of the surface will relax towards a state in which viscosity reduction under shear will become more marked, and thus the appearance of a new pseudopod in some other region of the cell will be favored.

It is appropriate to consider the phenomenon of contact inhibition, discovered by Abercrombie and Heaysman (1953, 1954 a). If two outgrowths of fibroblasts come into contact with one another, there is very little interpenetration of them. Soon after the first contacts of the outgrowths have been made, the cells in each are packed so closely side to side that if they are to continue moving in the same direction they must move over one another. But Abercrombie and Heaysman (1953, 1954 a) showed that this does not happen to any great degree. Abercrombie and Ambrose (1958) have found that when one fibroblast meets another head-on, the two leading edges appear to fuse forming a fairly strong adhesion. The undulations (ruffling) of the membranes are suppressed and soon afterwards the total movement of the fibroblast ceases: this is the phenomenon of contact inhibition. Very frequently new leading edges form in other regions of the cells after a while, and if they are not suppressed by contact with other cells they will initiate the movement of the cell off in other directions. The adhesion between the cells is broken after a while. The phenomenon of "contact retraction" (P. Weiss, 1958) appears to be the special case of contact inhibition when new pseudopods are formed in the free edges of the cells, but this need not always happen and the cells may remain in close apposition.

When the leading membrane of one fibroblast reaches the periphery of another one, their surfaces will start to interact. One of the cells will tend to move over the other, in consequence the surface undulations of one cell will be moving in the opposite direction from those on the other, and this will result in an increase of shear on the two membranes. The consequence is that the dynamic adhesiveness of the two surfaces will rise and this will lead to an adhesion, thus destroying the undulations in the surfaces. Even if the surface of one cell over which the other attempts to move is showing no movement, its adhesiveness would be raised by the shear of the other cell, whereas that of an inert surface like glass would be unaffected in a similar situation. After a while a relaxation will occur in the elements of the surface and the adhesion will weaken. This relaxation will permit the development of a leading pseudopod in some other part of the cell, if it is not inhibited by contact with yet other cells, and this will tend to draw the

two cells apart. Since the dynamic adhesiveness of fibroblasts is greater than their static adhesiveness, the moving leading pseudopods will be able to break the adhesion between the two fibroblasts. If there is no development of leading edges in some free region of the cell, there may be an attempt to renew movement in the region of contact between the two cells. Obviously this will be inhibited by the process described above. Thus the scheme accounts for contact inhibition. Abercrombie (1957b) has described in detail how these functions of contact inhibition will tend to promote the form shown by fibroblast outgrowths in tissue culture. The shear induced by contact with a following cell will tend to reduce the surface viscosity, and thus the hind end of a cell will tend to resemble the leading membrane in having a beating surface and being fan-shaped and spread, and this of course is so. But these features will be on a lesser scale, for if the hind membrane were able to reverse the movement of the cell it would destroy itself against the following cell by contact inhibition. It can be seen that the scheme of surface properties which was proposed explains many of the features of fibroblast appearance and behavior in tissue culture.

INTERACTIONS OF CELLS OF TWO TYPES

The most complete investigation of the interaction of two cell types in terms of their contact behavior has been that done by Abercrombie and Heaysman (1954b), and Abercrombie, Heaysman and Karthauser (1957) for fibroblasts and sarcoma. They found that cells such as mouse sarcoma 37, 180, etc. move easily over fibroblasts in tissue culture and that there appears to be no contact inhibition between the two types. This invasiveness in tissue cultures has been correlated with the malignancy of tumor cells in vitro (Abercrombie and Heaysman, 1954b). In explaining this, the same properties will be given to the fibroblast as before. Ambrose (1958) and Abercrombie (1957b) have suggested that sarcoma cells are less adhesive than fibroblasts. Coman (1944) found that malignant cells were less adhesive than normal cells, and L. Weiss (1959) reviews the large literature which supports this conclusion. This would correlate with a lower viscosity reduction under shear; in other words, sarcoma cells have a more condensed surface than fibroblasts. Nevertheless, since sarcoma cells are highly motile and fairly adhesive, their surfaces must lie in the region where there is appreciably viscosity reduction under shear. If figure 2 is examined again it will be seen that the surface conditions agreeing with highest motility are not those where the viscosity decrease is greatest but somewhat to the left. This is because the dynamic adhesiveness is large where the viscosity decrease is greatest, while somewhat to the left the adhesiveness has decreased much more than the viscosity has increased.

Consequently if sarcoma cells meet fibroblasts they will not undergo a viscosity reduction under shear such that their adhesiveness rises sharply, and there will not be formation of a firm adhesion between the two cell types. The forces of shear will reduce surface viscosity of the fibroblast considerably, and thus tend to aid its own motion. If it were in contact with another

fibroblast this would be opposed and destroyed by the adhesion between them. But it takes two surfaces to make an adhesion, and since the sarcoma cell will not be highly adhesive the adhesion formed with the fibroblast will be too weak to oppose the motion of either surface appreciably.

A very contrary example is found in the interaction of fibroblasts and epithelial cells. It has been found (Curtis, unpublished) that there is almost no trace of overlap between these two types of cell, which suggests that contact inhibition is completely expressed in this case. If it is assumed that epithelial cells are very adhesive and show little or no viscosity decrease under shear, then fibroblasts coming in contact with them will be held so strongly that further advance of one cell type over the other will be impossible.

REAGGREGATION

The ability of embryonic cells to come together (reaggregation) after their separation and build up an organized body has been known since the work of Roux (1894). Reaggregation of mixed types of cells, such as whole embryos, involves two distinct mechanisms (Lucey and Curtis, 1959). Firstly, the cells lying in juxtaposition adhere to form clumps, and secondly, the cells in these clumps reassort so that cells of like type lie beside one another. The second process has been considerably studied by Galtsoff (1925 a, b), Lehn (1953), Moscona (1956, 1957) and Wilson (1907). Holtfreter described the first part of the process in amphibian material (1947 a, b); Stefanelli and Zacchei (1958) have made a similar study in chick embryo material; Curtis (1957 a) and Lucey and Curtis (1959) have made a time-lapse film study of the first stage of the process in Xenopus embryos. Chemical studies of the process (Curtis, 1957 b) suggest that calcium, which is essential for reaggregation, is bound to the cell surface, where it presumably acts by reducing the surface charge sufficiently for the cells to become adhesive. If the cells are in a medium containing calcium ions, the hyaloplasmic margins of the cells move rapidly around the periphery of the cell: the cyclosis described by Holtfreter (1943 b, 1946). A similar phenomenon was noted by Stefanelli and Zacchei (1958) in chick material. If the cells are fairly close together the hyaloplasmic margins will frequently come in contact. The first adhesions form where these margins meet, but the margins may be in contact for an appreciable while before an adhesion forms. The cells have no motion over the substrate before an adhesion is formed.

When embryos are disaggregated by removing calcium and magnesium with chelating agents, the surface charge of the cells will rise and this will cause them to come apart. Even when calcium and magnesium ions are returned to the medium in which the cells are growing, there is no motion of complete cells. This suggests that the cell surface is in some way unsuitable for movement over the substrate. The rotating cell margin involved in cyclosis hardly touches the substrate but moves mainly on the free surface of the cell, Curtis (1957 a). This can be explained by presuming that the surface is of such packing (that is, surface area per element) that it under-

goes viscosity increase under shear and is of very low adhesiveness. In consequence any movement of the cell surface in contact with the substrate will undergo a shear, which opposes it by a viscosity increase. But movements on the free surface of the cell will not be opposed by so great a shear for they are moving against a fluid medium. On disaggregation the surface charge rise will be accompanied by a rise in surface pressure (Davies, 1951 a) and this will be accompanied by a rise in surface viscosity (Isemura and Hamaguchi, 1954). When calcium or magnesium ions are returned to the surface the surface charge will be lessened. But the conversion of the surface from one packing to a less compact one will require an energy of activation. In consequence the surface will revert to its expanded state at a rate dependent on the magnitude of this energy barrier. Since adhesions are not formed for some while after calcium etc. are returned to the medium, it is presumed that the reversion of the surface is a slow process.

As the hyaloplasm margins move against one another in cells that are in contact, periodically small regions of the cell surface that have reached the more expanded state will pass across the contact. As time passes there will be more and more of these. If such a region on one surface coincides with that on the other an adhesion may form, because of their greater surface area per element. Since the adhesion will deform the cell surfaces from their spherical state locally, there will be a considerable increase of surface area close to the adhesion. This expansion of the surface will cause unexpanded elements to pass into the expanded state much more easily, and an adhesion will spread very rapidly over a large region of contact. This very rapid spread of an adhesion from its point of initiation was described by Lucey and Curtis (1959).

These considerations raise the question of the converse nature of cell behavior in tissue culture and in reaggregation. Tissue culture cells tend to move away from the region of greatest population, reaggregating cells move towards it. There is no evidence that reaggregating cells of metazoa are attracted towards one another (Kuhl, 1937; Lucey and Curtis, 1959) except when they are already in contact. Likewise contact inhibition does not act to space cells out unless they are in contact.

If the surfaces have an area per element such that the surface viscosity decrease under shear is considerable—that is to say, if they lie to the left-hand side in the region of viscosity decrease under shear in figure 2 (see letter A therein)—then the following behavior will result: they will have a low static and a fairly low dynamic adhesiveness. Shear will promote the motion of the cells, and the increased shear between two cells will greatly promote their motion on one another. The dynamic adhesiveness will not be large enough to cause contact inhibition. But the fall in shear on parts of the cell, which move off another cell onto the substrate, will raise the viscosity so that motion on the substrate is less favored. In fact a form of "contact promotion" will result. The consequence of this form of behavior will be the forming of compact bodies of cells with a minimum of surface in contact with the substrate. This is of course what happens in reaggregation

(Holtfreter, 1943 a, b, 1944; Townes and Holtfreter, 1955; Lucey and Curtis, 1959). Thus it can be seen that the phenomena of the first stage of reaggregation are adequately explained by assuming that at first the cells have surfaces of a compressed nature, which become sufficiently expanded for shear to produce a large enough viscosity decrease for active motion of the cells over one another.

SPECIFIC ADHESION

The sorting out of cell types in reaggregation (Townes and Holtfreter, 1955), the similar phenomena in wound healing (Chiakulas, 1952; Hooker, 1930; Abercrombie, 1957 a), these and many other observations require an explanation that cells in some way adhere preferentially to their own type. It will be assumed that the tendency towards surface expansion which was used to explain the first stage of reaggregation continues in the second stage. The cells will pass into a state like that of cancer cells, and then into a fibroblast condition. Suppose that one cell type is the first to increase its surface area per element. As this happens its static adhesiveness will rise but at first it will remain comparatively mobile. In this state it will move actively around inside the reaggregate as will the other cells. But by reason of its more expanded surface than the other cells it will show a greater static adhesiveness than they, and shear will not promote its mobility to such a degree as in the other cells. In consequence those cells of this type which arrive at the surface by random movement in the reaggregate will tend to remain there. In a reaggregate this cell type which accumulates at the periphery appears to be the ectoderm. In consequence of its being the first type of cell to alter its surface to this more adhesive condition, it might be expected to be the first type of cell to lose the ability to begin reaggregation at the start of the first stage where low static adhesiveness and high viscosity decrease are required. Experiments on the reaggregation of Xenopus embryos have shown this to be the case (see figure 3).

If a second cell type alters its surface properties so that it becomes of larger area per surface element, it will become less motile and more adhe-

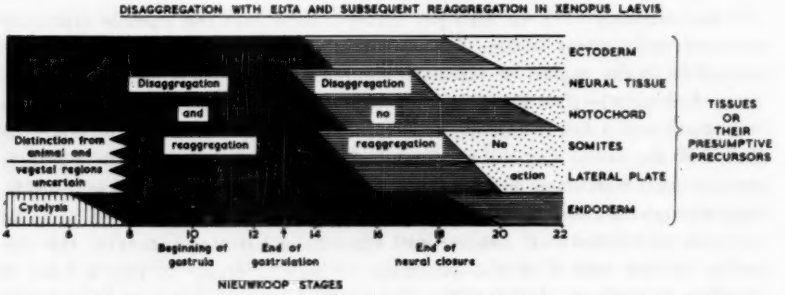

FIGURE 3. The age (in Nieuwkoop stages) of the various tissues of the Xenopus embryo are shown here in relation to their ability to be disaggregated and to reaggregate. The ability to reaggregate dies out progressively from ectoderm to endoderm.

sive and will begin to be able to show contact inhibition. Suppose that this alteration occurs after the change of the first type is well under way. It will move about in the center of the reaggregate easily and randomly, for while in contact with the unchanged cells it will not undergo inhibition, just as fibroblasts and sarcoma cells do not hinder each others' movements. But if it comes in contact with the ectoderm layer, which will by now be in such a state that it is able to show considerable contact inhibition, it will be immobilized. Any attempt to move back into the region of unchanged cells will be prevented in the same way that ectoderm cells were stopped from reentering the reaggregate earlier. Consequently, a layer of cells of this second type will be built up just inside the ectoderm. If each cell type changes in succession a series of layers of cells, each layer of one type will be built up in the reaggregate. The outermost layer will have been the first to change, the innermost the last. The experiments of Townes and Holtfreter (1955) show that this successive layering is in fact found in reaggregates. Moreover, the results of my experiments on the reaggregation of Xenopus embryos show that the various cell types lose the ability to reaggregate from the very beginning in the same order that they are found passing inwards in the reaggregate (see figure 3). And this loss of the ability to reaggregate from the beginning suggests the change of the surface from a compressed one to a fairly expanded one.

It might be assumed that all cell types finally arrive at the same surface state in this scheme. However, this restriction is not necessary, provided that the last cell type to change shows sufficient contact inhibition with the layer outside it to prevent invasion. Since cells show different behavior in tissue culture, according as to whether they are fibroblasts or epithelia, it would seem best to assume that the various cell types in complete reaggregates are in different surface states but that all have sufficiently expanded surfaces to show appreciable contact inhibition.

Moscona (1956, 1957) found that in reaggregates of cells of two specific types, cells become grouped according to their histological type and not according to their species type. Studies of sponges by Galtsoff (1929) and Laubenfels (1927) had suggested that the reaggregation of cells of two species mixed together led to groupings of cells according to their species type. On the present scheme these contrary results can be reconciled. If the changes in the reaggregating cells proceed at about the same speed and the same order in each species, the cells will tend to group according to their histological type. If they proceed much more rapidly in one species than the other, there will be a separation of the cells of each species. A gradation of type of separation is to be expected; some pairs of species should show a mixture of the two types of grouping.

No attempt has been made to explain phagocytosis, pinocytosis, or sperm and egg interactions. These phenomena, which can be specific and in which adhesion acts, are very different from the forms of behavior which have been described, for here the surfaces come into such close proximity that the sperm or phagocytosed particle is included into the cell. Besides, in these

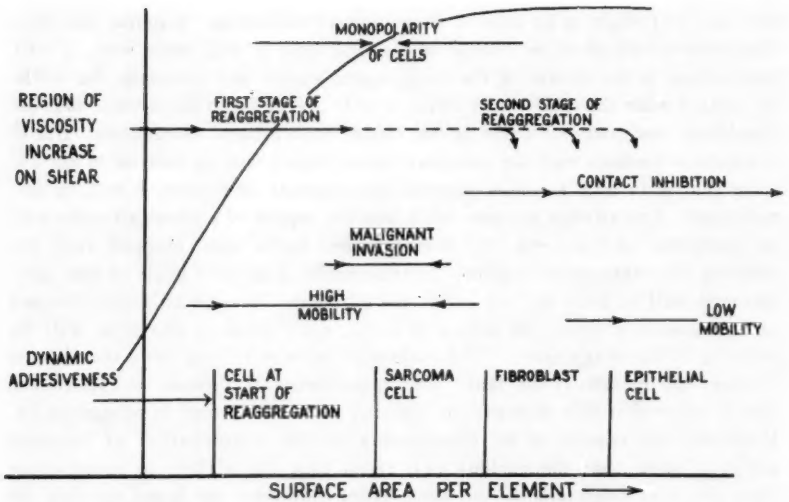

FIGURE 4. This diagram shows the right-hand side of figure 2 reproduced with the addition of the various forms of cell behavior in relation to the nature of the surface. Cell types are indicated in roughly those positions whose physical properties account for their behavior. 'Cell at start of reaggregation' indicates condition of cell when first adhesions have formed and movement is about to start.

cases unlike surfaces come into contact, not like ones, and there is generally the inclusion of a small object into a large cell; this is greatly favored by Brownian movement enabling the small particle to surmount the potential energy barrier to close interaction. For these forms of cell contact the theory of the interaction of unlike surfaces, with in some cases the possibility of antigen-antibody type reactions in adhesion, may provide a satisfactory explanation. The types of cell behavior which have been considered and their relation to physical properties are expressed diagrammatically in figure 4. The chief importance of the scheme which has been used for explaining cell behavior is probably its general form, not its precise nature. It is possible that elastic rather than viscous properties are to be preferred, in which case shear would alter the elastic properties of the surface.

SUMMARY

The rheological and adhesive properties which the cell surface may possess have been adduced from the known physical and chemical properties of the surfaces of cells and artificial systems. It is shown, using the Verwey-Overbeek theory of the interaction of like surfaces, that cells will form stable adhesions when separated by 100-200 Å, without there being any cementing material between them, though its existence is not precluded. On physical grounds it is shown that the form of interaction of the surfaces proposed for adhering cells by Weiss, Steinberg, and others is unlikely to occur. However, these schemes may explain the interaction of unlike sur-

faces, for example, sperm-egg relations and phagocytosis etc. Consideration of the chemistry of surfaces suggests that the non-newtonian viscous properties of the surface and its adhesiveness are closely related. On this basis a set of physical conditions have been proposed for various cell surfaces, and it is shown how these would produce the types of contact behavior which have been described for them. For fibroblasts the phenomenon of contact inhibition, the mode of movement, and their tendency to being monopolar have been examined. By proposing slightly different properties for sarcoma cells, reaggregating cells, and epithelia, it is possible to account for the types of contact behavior which they show. An explanation for the specific adhesion of like cell types is advanced.

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INTRODUCTION TO SYMPOSIUM ON THEORETICAL RADIOBIOLOGY*

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Theoretical work has always been an important part of the study of radiobiology, but it has never been needed more than at the present time. Propositions that seemed well established some years ago have become questionable but have not been replaced by new ones; new experimental methods have been developed that are highly sophisticated but require specialized skills and equipment; and new data have been accumulated but not yet organized sufficiently for clear presentation. Also, there is a growing tendency to partition radiobiology into a number of highly specialized disciplines which is not altogether fortunate; this might be counteracted by the integrating effect of theoretical work.

This symposium was arranged by Professor Ernest Pollard. The papers are within the area covering macromolecules up to entire cells. Although theoretical radiobiology extends to the higher levels of organization (for example, Kaplan's theory of tumor induction and Sacher's stochastic theory of lethality), it was obviously impossible to cover all of theoretical radiobiology in a single session.

Theory is most urgently needed when there is either a dearth or an overabundance of data, the problem being either to obtain the maximum information from sparse data or to select the relevant material out of a vast accumulation. The field of cellular and subcellular radiobiology is in the latter category, and it includes at least four well-defined levels of organization containing individual structures differing in size by more than ten orders of magnitude. Every biological macromolecule is affected by radiation in at least two distinct ways, and probably in several; however, only a small fraction of the changes is expressed at the next higher level of organization, that of organelles and organelle systems. Many changes of organelles are known, but only a fraction of them seems to affect the viability and function of the entire cell. It is one of the basic problems of radiobiology —possibly *the* basic problem—to sort out, among the changes known to occur at a given level of organization, those that affect the next higher level, and, conversely, to recognize in a given biological response the underlying changes at lower levels of organization.

*Presented at the Symposium on Theoretical Radiobiology of the Radiation Research Society held at Pittsburgh, Pa., May 20, 1959.

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Theoretical radiobiology is two-sided: what is known about the organization of living things determines the analysis of how radiation effects are propagated throughout the organization, and what is known about the propagation of radiation effects helps to elucidate the pattern of organization. This two-sidedness is illustrated in every paper of this symposium. Hutchinson's study of two classes of radiation effects on macromolecules, separately and conjointly in the cell, yields a definite condition on propagation of molecular effects to the cellular level and, at the same time, information on the manner in which certain macromolecules are imbedded in the cell. Pollard, putting together recent results on radiation effects and recent concepts of the normal synthetic processes in bacteria, develops a novel and highly specific theory of lethal effects in bacteria which also is a theory of the interrelations between synthetic processes in the cell. Wolff's investigation of the healing of radiation-induced chromosome breaks throws light not only on an important kind of radiation damage but also on the structure of chromosomes. The same interplay between study of radiation effects and study of biological organization pervades the two papers which attack the problem of radiation effects on the cell from opposite directions: Puck's paper emphasizing the quest for the basic mechanisms underlying cell death, Errera's the vast variety of responses which can be elicited in the cell.

Theorizing, as exemplified by the papers in this symposium, is hard work, often tedious, and rarely resulting in striking generalizations. It is neither as pleasant as meditating nor as glamorous as attending conferences, activities often falsely confused with theorizing. Both activities aim at deriving new propositions from old facts, and so does theorizing; but for theorizing, rigorous logico-mathematical methods are used. Logical manipulations require well-defined abstractions. Every abstraction is achieved at the cost of neglecting some features of the observations. There is no calculus, no set of reliable mechanical rules, by which it can be determined in advance which features are important and which negligible. Hence, no theory is better than the abstractions on which it is based. Furthermore, no theory can be better than the observations on which the abstractions are based. These circumstances do not excuse the theorist from using the utmost care in formulating a theory, but he must be willing to discard it and replace it with a new and better one as new and better data become available. Great and lasting theories are few and far between, but those that stimulate the very work that causes them to be quickly superseded are also useful. In this sense, the contributors to this symposium will be satisfied if their statements have to be modified a few years hence.

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RADIATION INACTIVATION OF MOLECULES IN CELLS

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Radiation can act on the cell either by destruction of important molecules, or by a disruption of the way in which these important macromolecules are organized into functional units. This paper will be concerned with the first item, the destruction of molecules. A word should be said, however, on the possibilities of radiation as a way of creating disorder in a cell without necessarily disrupting a macromolecule, lest the impression be given that radiation effects are always due to molecular destruction. This is by no means necessarily the case, and there is much which suggests the contrary. For example, the breaking of a chromosome appears to require the simultaneous production of 15–20 ion pairs by a charged particle within a particular chromosome (Lea, 1955). The apparent necessity for these ionizations to occur simultaneously argues for the possibility that the breakage is produced by the cleavage at the same time of a number of secondary bonds, bonds which if broken individually would rapidly rejoin. A possible mechanism for such secondary bond breakage might be that suggested by Platzman and Franck (Platzman and Franck, 1958). Here secondary bond breakage is ascribed to the wave of polarization which spreads out from the site of an ionization produced, essentially instantaneously, in a medium containing large permanent dipole moments. We will not, however, consider any farther such disorganizing processes, but only those which lead to changes in the structure of individual macromolecules.

To understand the manner in which radiation affects molecules located within a living cell, we would like to believe that the processes taking place are those which have been recognized in studies on various preparations of biological molecules. These are direct action, in which an ionization occurs directly within the structure of the molecule itself, and indirect action, in which reactive species created in the surrounding medium, mainly water in the cell, diffuse to the macromolecules and react chemically.

DIRECT ACTION

Direct action may be investigated by radiation studies on biological materials in a dry state. Such studies were initiated by Lea (Lea, 1946). More extensive studies have since been carried out, mainly by the Yale group, and many of the results have been summarized by Pollard, Guild, Hutchinson and Setlow (1955).

Although ionizing radiation creates both excitations and ionizations, it is clear that ionization is the principal agent in causing molecular inactivation.

*Presented at the Symposium on Theoretical Radiobiology of the Radiation Research Society held at Pittsburgh, Pa., May 20, 1959.

It is well-known that the number of biological molecules inactivated by ultraviolet in the wavelength range 2000-3000 Å is only one per cent or less of the number of photons absorbed, as opposed to an efficiency of the order of unity for ionizing radiation (Setlow, 1957). Recently Setlow (Setlow, Watts and Douglas, 1959) has extended the yield measurements down into the vacuum ultraviolet. Down to almost 1200 Å the efficiency is almost constant, and is about equal to that at 2537 Å. At about 1200 Å the efficiency shoots up rapidly by an order of magnitude. Since 1200 Å photons have an energy of 10 ev, and since the ionization potential of proteins is the order of 10 ev, it is reasonable to assume that this rapid rise in efficiency is due to ionization. This conclusion is in agreement with earlier work in which it was shown that very low voltage electrons were very inefficient in inactivating protein monolayers, but that a rapid rise in efficiency was found as the electron accelerating voltage was increased above 10 volts (Hutchinson, 1954).

The many experimental results on radiation effects on dry molecules can best be summarized by saying that inactivation is caused by one or more ionizations occurring in a target volume which is approximately the size and shape of the molecule being irradiated. For sparsely ionizing radiation such as high speed electrons (figure 1a), the ion clusters can be considered to be produced at random throughout the irradiated material, and target theory predicts that the relationship between the target volume, expressed in molecular weight units, and D_{37} (the dose resulting in 37 per cent survival) given in rads will be approximately

$$(MW)(D_{37}) = .72 \times 10^{12} \tag{1}$$

For heavily ionizing radiation such as fast positive ions, as shown in figure 1b, the radiation sensitivity is determined by the cross-sectional area

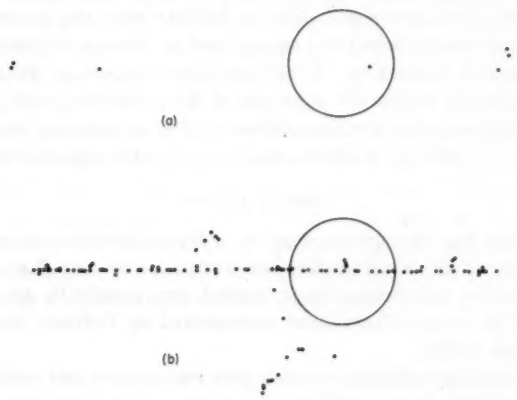


FIGURE 1. The distribution of ionizations in space for (a) sparsely ionizing radiation such as fast electrons and (b) heavily ionizing radiation such as positive ions.

TABLE 1

Target size with different radiations for dry trypsin

Radiation	LET	Target diameter
Gamma rays	3 ev/100 A	36.6 A
4 Mev deuterons	240	38
120 Mev carbon ions	2200	38
160 Mev oxygen ions	3900	38

of the target volume (Pollard, Guild, Hutchinson and Setlow, 1955). The applicability of target theory to direct action depends partly on the important fact that the same target volume is found with various types of radiations, as shown in table 1.

In figure 2 the apparent target molecular weight is plotted against the physico-chemical molecular weight. The straight line is the theoretical curve for the case in which the two are equal, and is seen to be a remarkably good fit over a range of molecular weights varying by a factor of 10^5. The systematic deviation of the experimental points from the line at low molecular weights can readily be explained since the target calculations were all carried out under the assumption that the *ion cluster* is the unit of inactivation. For small enough molecules, the finite separation of the ions in a cluster would readily explain the observed deviations.

The excellent general agreement should not be taken to mean literally that only an ionization inside the molecule will cause inactivation, and that an ionization outside will under no circumstances have an effect. For one thing, some of the points in figure 2 differ from the curve by more than experimental error. The temperature of irradiation can also have an effect;

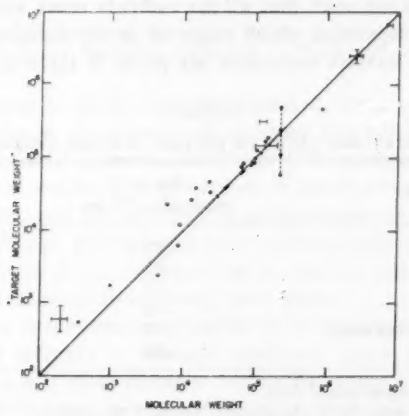


FIGURE 2. A plot of apparent target molecular weight against physico-chemical molecular weight. The data plotted are largely that of Table II, in Pollard, Guild, Hutchinson and Setlow (1955).

TABLE 2

Relative radiation sensitivities of enzymes in the dry state
(Braams, Hutchinson and Ray, 1958)

Additive	Invertase	Ribonuclease	Papain	Urease
Pure	1.0	1.0	1.0	1.0
Cysteine	0.5	...	...	0.5–0.7
Glutathione	0.5	0.52 ± 0.10	...	...
Yeast extract	0.45	0.52 ± 0.10	0.6–0.8	0.8
Ribonucleic acid	...	0.41 ± 0.07		
Acetate buffer	1.4–1.6	4.8 ± 1.4		
Sucrose		2.6 ± 0.5		
Sodium chloride	1.5–3.5	0.98 ± 0.21		

the four points in figure 2 connected by a dashed vertical line represent the variation in sensitivity of the dry enzyme catalase from −196°C (bottom point) to +110°C (top point). Furthermore, the material surrounding the dry enzyme can also change the apparent target volume, as shown by the data in table 2. Nevertheless, the basic radiation sensitivity of dry biological molecules seems to be determined pretty much by their molecular weight, with significant but not order of magnitude changes caused by various factors.

INDIRECT ACTION

Eighty per cent of a living cell is water, and the irradiation of a cell will produce large quantities of highly reactive substances such as H, OH, and H_2O_2, which can diffuse to the surfaces of macromolecules and alter them by chemical combination, which may or may not inactivate. Probabilities of inactivation by this means are customarily given in terms of the number of molecules inactivated by the radicals produced by some convenient unit of dose, under conditions such that all the radicals react with the molecules, and not with any impurities which might be in the solution. Some typical yields Y for some enzyme molecules are given in table 3, with the unit of

TABLE 3

Typical ionic yields for enzymes in dilute solution

Enzyme	Yield molecules/33 ev	Reference
Invertase	.05	*
Catalase	.03	†
Cytochrome C	.10	‡
Ribonuclease	.48	§
Alcohol dehydrogenase	.93	†
Coenzyme A	.90	§

*F. DeFilippes, unpublished data.

†E. S. G. Barron, Ann. N. Y. Acad. Sci. 59, p. 578 (1955).

‡H. Rajewsky, G. Gerber and H. Pauly, Adv. in Radiobiology, p. 25. Oliver and Boyd (1957).

§F. Hutchinson and D. A. Ross, Radiation Research 10, p. 477 (1959).

dose being taken as the absorption of 33 ev, or about one ion pair. The values given may be considered typical.

INACTIVATION OF ENZYMES IN A CELL

The contributions of the direct and indirect action to the inactivation of enzymes in a cell may be assessed by irradiating cells in both a wet and in a dry state, extracting the enzymes, and determining the activity of each by a suitable assay. Such experiments always show an exponential decrease in enzyme activity with dose. In table 4 are tabulated some typical results. The most significant points are the large doses required, and the wide variation in the ratio of doses from wet to dry.

TABLE 4

Radiation inactivation of enzymes in cells D_{37}, megarads

Enzyme	Cell	Wet	Dry	ρ, Angstroms	Reference
Invertase	Yeast	6	12	29	*
Alcohol dehydrogenase	Yeast	1.3	28	31	*
Coenzyme A	Yeast	3	200	35	*
Coenzyme A	E. coli	15	...	17	†
Coenzyme A	Peas	5	...	30	†
Coenzyme A	Beef heart	100	...	5	†
	Beef liver	100	...	5	†
Acetylcholin-esterase	Electric organ of eels	4.8	4.8		
	Human red blood cells	4.8	4.8	0	‡
	Rat brain homogenate	4.8	4.8		

*F. Hutchinson, A. Preston, and B. Vogel, Radiation Research 7, 465–472 (1957).
†F. Hutchinson and C. Norcross, Radiation Research, in press.
‡G. Cotzias and I. Serlin, Radiation Research 7, 55–66 (1957).

Looking, for example, at the first three entries, we see that the ratio of wet to dry sensitivity in the yeast cell is about 2 to 1 for invertase, about 20 to 1 for alcohol dehydrogenase, and 100 to 1 for coenzyme A.

To interpret these results it is convenient to ascribe the sensitivity when dry to direct action, and the increase in radiosensitivity when wet to indirect action. Although this appears to be a reasonable assumption, there is actually very little direct evidence for it, and its postulational nature must be borne in mind when interpreting the results.

Within a cell the concentration of solids is so high that the migration of radiation-produced radicals is strongly inhibited, since the radicals produced will travel only a short distance before they react with a molecule of some kind. This factor can be taken into account by a method first used by Zirkle and Tobias (1953). In this model, the radical is assumed to move through a medium in which it has a probability a of reacting with a molecule in unit time. If the molecule is formed a distance r from the center of a

spherical target molecule of radius r_o (see figure 3a) then the probability that the radical will react with this specified molecule before it is removed by another reaction is given by

$$q \frac{r}{r_o} e^{-(r - r_o)/\rho} \tag{2}$$

where

$$\rho = \sqrt{\frac{d}{a}} \tag{2a}$$

d = diffusion coefficient of the radical

q = a parameter which measures the probability of a reaction between the radical and molecule at an encounter.

For unit probability, q = 1, and as the probability decreases, so does q.

The parameter ρ is a measure of the action radius of the radical in the particular medium. It can readily be shown that under this assumption the fraction S of a specific kind of molecule surviving a dose D can be written

$$S = \exp - [4\pi r_o^2 \rho(1 + \rho/r_o) Y q] D \tag{3}$$

where the dose D is expressed in ion pairs per unit volume.

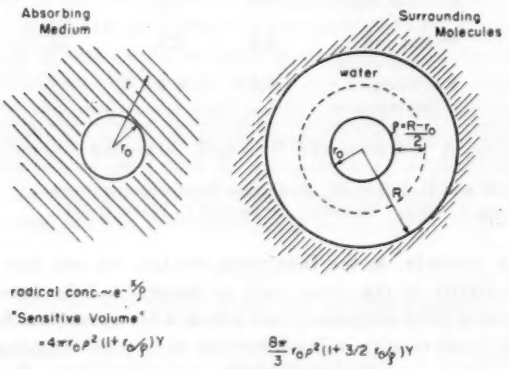


FIGURE 3. A diagram showing the magnitude of the indirect effect to be expected in the cell. The left side (a) shows the case for a medium in which the radicals formed by radiation are absorbed in the medium; the right side (b) shows the case in which all absorption takes place on macromolecules forming the boundaries of the water solution within which the radicals are formed.

An alternative formulation is shown on the right-hand side of figure 3. Here the radicals are assumed to be formed in the water layers between the cell macromolecules. If the parameter ρ is allowed to equal the thickness of water surrounding each macromolecule, then the survival curve becomes

$$S = \exp - \left[4\pi r_o^2 \rho \left(1 + \frac{3}{2} \rho / r_o\right) Y q \right] D \tag{3a}$$

which is very similar to expression (3) derived on the other assumption.

Including the direct effect for a molecule of sensitive volume V, the survival after a dose D expressed in ion pairs per unit volume will be

$$S = \exp - [V + 4\pi r_o^2 \rho (1 + r_o/\rho) q Y] D \tag{4}$$

The magnitude of V is determined by the inactivation in the dry state. In all cases for the enzymes in table 3 the volumes determined by the dry irradiations are in reasonable agreement with the known molecular weights and with irradiation studies on purified enzyme preparations. The experimental values of the D_{37} for wet irradiations are then combined with the known values of V, r_o and Y and an assumed value of q equal to unity to calculate ρ, the mean action radius for radiation-produced radicals in the cell, which are tabulated in table 4.

The most significant fact about the values of ρ is the small values, of the order of 15-35 Å, which are found necessary to explain the indirect action observed. It also should be noticed that the widely varying ratios of indirect to direct action observed for three different enzymes in yeast cells are all accounted for by an approximately constant value of ρ. Finally, it seems that some molecules, such as coenzyme A in beef heart and liver cells, and acetylcholinesterase, have apparently greatly restricted access to water, since the presence of water does not increase the radiosensitivity in the cells to any marked extent.

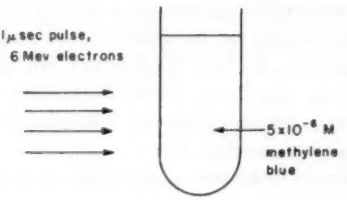
An interesting correlation which can be made here is with the average thickness of water which surrounds a macromolecule in the cell. This thickness depends somewhat on the arrangements of macromolecules and water assumed in the cell, but various assumptions lead to mean thicknesses the order of 20–40 Å. From the general agreement with the action radii of radicals determined from the radiation data, a relatively simple picture can be drawn: the inactivation of a molecule in a cell is considered to be due either to an ionization produced within the molecule, or to a radical produced in the water immediately surrounding the molecule, which diffuses to the molecular surface and reacts.

THE PROBABILITY OF A REACTION IN A RADICAL-MOLECULE ENCOUNTER

The simple picture given above depends on the assumption that a radical reacts with a molecule on its first encounter. That is, the value of q in equations (2-4) was assumed to be unity. This assumption is not perhaps as drastic as it might seem at first, since a radical, once it first meets a molecule, will, on the average, make many collisions with it because of the caging effects of the surrounding molecules.

An experiment which tends to support this reaction on first encounter is shown in figure 4. A one-microsecond-pulse of electrons was used to create, effectively instantaneously, a certain concentration of radicals. The radi-

cal concentration then decays by reaction with molecules present in solution. The radical concentration calculated is the OH concentration, using the accepted yield of 2.16 OH radicals per 100 ev absorbed in water. It is seen that only when the initial OH concentration somewhat exceeds the methylene blue concentration is the number of methylene blue molecules decolorized by a given dose reduced by radical-radical encounters. A simple analysis (Hutchinson, 1958) shows that the data are consistent with the assumption that reactions in radical-radical and in radical-molecule collisions are about equally likely. This strongly implies that collisions take



Dose pulse	Radical Concentration	D_{37} Dose
.001 Kr	.003 x 10^{-6} M	8±1 Kr
.9	2.	8±1
2.3	6.	8±2
6.6	16.	16±4
13.3	35.	42±5

FIGURE 4. The results of irradiating a dilute solution of methylene blue with one microsecond pulses of electrons. The observed D_{37} increases, because of radical-radical recombination, as the dose per pulse, and therefore the instantaneous radical concentration, increases.

place on each encounter of an OH radical and a molecule. The extension of this single experimental result to the case of collision of radicals with all kinds of organic molecules is a considerable extrapolation, but is supported by the agreement between the radical action radius from radiation data, and the water thicknesses which must exist around an average macromolecule in a cell.

INACTIVATION OF DEOXYRIBOSENUCLEIC ACID IN A CELL

We have recently studied the inactivation of deoxyribosenucleic acid (DNA) in irradiation of intact cells, with the results shown in figure 5. The radiation effect on the DNA was assayed by measuring the ability of DNA extracted from irradiated pneumococcus cells which are resistent to streptomycin, in moderate concentrations, to transform sensitive cells by the well-known transformation process.

The three-fold increase in radiation sensitivity in going from (anoxic) dry irradiation to wet anoxic conditions can be accounted for by a radical action radius of about 10 Å, if the DNA is considered to be a long thin cylinder of radius 10 Å. The decrease in sensitivity in the irradiation of lysed cells is probably real, reflecting some small change in radiation sensitivity of dry DNA with the change of the surrounding materials.

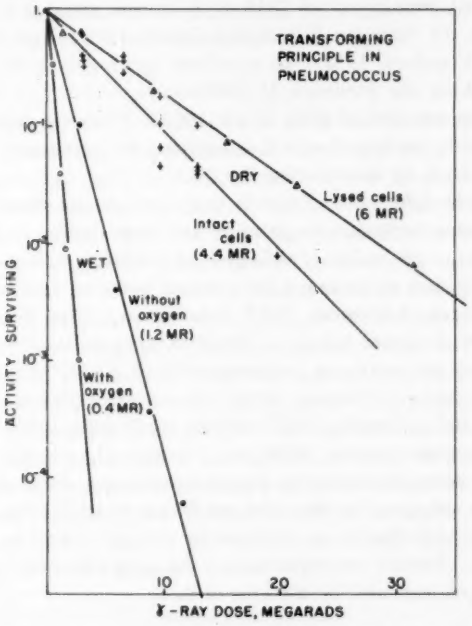


FIGURE 5. The inactivation of the transforming ability of DNA extracted from pneumococcus cells (streptomycin resistant) with radiation. The conditions under which the cells were irradiated are indicated on each curve. The irradiation of cells in one per cent cysteamine in the presence of oxygen led to an inactivation curve which agreed within experimental error with the irradiation of wet cells in an anoxic state. All dry data are for irradiation in the anoxic state.

OXYGEN EFFECTS IN CELL IRRADIATIONS

When pneumococcus cells are irradiated wet and in the presence of oxygen, the DNA is three times as sensitive (figure 5) as for anoxic irradiation. Both Howard-Flanders (1958) and J. G. Van Dyke (personal communication) have found that when the DNA from T-2 bacteriophage is irradiated in the *E. coli* cell directly after phage attachment and DNA injection, there is an oxygen effect which increases the sensitivity to radiation by a factor of two. The indirect action on DNA has been shown (Ephrussi-Taylor and Latarjet, 1955; DeFilippes and Guild, 1959) to be insensitive to the oxygen concentration. Guild (1959) has shown that the direct action of ionizing

radiation on dry DNA is probably increased in the presence of oxygen, although the factor by which the sensitivity is increased is not yet clear. Suppose the direct action could be increased by a factor of three in the presence of oxygen, a factor which is consistent with Guild's data; then the deduced increase in sensitivity in the cell (taking into account the lack of an oxygen effect for the indirect action) would be 1.7, or less than the observed effect.

The increased sensitivity of DNA with oxygen tension is in excellent agreement with the increase in radiosensitivity with oxygen found for many cells (Howard-Flanders, 1958). In excellent agreement is the protection afforded the DNA by the presence of cysteamine, which also affords similar protection to the survival of cells (Patt, 1955). Thus the data available are in agreement with the hypothesis that ionizing radiation acts on the ability of a cell to multiply by inactivating the DNA.

It should not be inferred that this is at all conclusive, however, since the effects of ionizing radiation on proteins are very similar to those on DNA. The direct effects of ionizing radiation on enzymes have been shown by several investigators to increase by a factor between two and three in the presence of oxygen (Alexander, 1957; Hutchinson, 1958; Shalek and Gillespie, 1959). On the other hand, no effect of oxygen has been found on indirect action on the enzymes carboxypeptidase (Dale, Gray and Merideth, 1949), ferricytochrome C (Barron, 1955), ribonuclease (Holmes, 1950), chymotrypsin (Moore and McDonald, 1955), trypsin (McDonald, 1955), catalase (Sutton, 1952), lysosyme (Shalek, 1959), and creatine phosphokinase (Anderson, 1958). It has been reported that deoxyribonuclease does show an oxygen effect in dilute solution, but the data are fragmentary (Okada, 1957). Measurements on oxygen effects on enzymes in the cell would be most interesting, but in their absence we might hazard the guess that the increase in wet cells with oxygen may well be a factor of three.

CONCLUSIONS

A broad conclusion which can be drawn from the material which has been presented here is that very large doses, of the order of megarads, are needed to reduce substantially the activity of any specific type of molecule in a cell. Thus it appears probable that if the inactivation of the cell's ability to divide is due to destruction of individual molecules, then there must be, in the cell, many molecules of which *all* must function in the process of division.

A second conclusion which is closely connected with the first is that the inactivating events initiated by ionizing radiation tend to be pretty much localized. This tends to lend a renewed significance to the concepts of the "target theory" of the action of ionizing radiation.

Thirdly, it may be said that a reasonably satisfactory picture of the way in which ionizing radiation acts on the structure of the molecules in a cell can apparently be given. In this view, the inactivating event is the occurrence of an ionization within the molecule, or within the water layer sur-

rounding the molecule. The one feature which this picture does not as yet fit quantitatively is the increased sensitivity in the presence of oxygen. There are possible simple extensions of this picture which may conceivably solve this problem.

And finally, the inactivation of DNA molecules in the cell is seen to parallel the known radiosensitivity of cell survival as measured by ability to divide. The significance of this is not yet clear, however, since it is quite possible that the inactivation of enzymes in cells and of DNA may vary with conditions at the time of irradiation in the same way.

SUMMARY

The mechanisms by which molecules in cells are inactivated by ionizing radiations are discussed. Experiments on the inactivation of enzymes and of desoxyribosenucleic acid in dry cells are in good agreement with the hypothesis that direct action requires an ionization either within the molecule, or very close to it. When wet cells are irradiated, the increased radiosensitivity can be explained quantitatively by assuming an indirect effect caused by the diffusion of radiation-produced radicals which diffuse a distance the order of magnitude of 30 angstroms. These mechanisms explain the experimental fact that biological macromolecules in cells are very resistant to radiation, and require doses the order of megarads, or greater, to inactivate half of the molecules of a given kind. They also imply that the initial biochemical effects are produced very close to the sites of the ionizations, providing a justification for the use of target theory. It thus appears that if the inactivation of a living cell is to be ascribed to the inactivation of molecules, then a number the order of a thousand molecules must be assumed, each indispensable for the process of cell division, to account for the observed effective doses.

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THEORETICAL ASPECTS OF THE EFFECT OF IONIZING RADIATION ON THE BACTERIAL CELL*

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INTRODUCTION

Ionizing radiation effects on bacterial cells have received considerable attention and many aspects of cell behavior have been found to be influenced by x-rays, gamma rays, fast electrons and heavy fast particles such as deuterons or alpha particles. No simple, conclusive theory of radiation action has yet been worked out, though various suggestions have been made. One relatively new aspect of modern knowledge, the hypothesis that the operation of a cell is determined by a relatively limited set of molecules, for example, of DNA, RNA, protein and lipid, can be invoked. One would accordingly think that the results quoted by Hutchinson (1960) in this symposium would, together with modern bacterial cytology, suffice to explain the nature of radiation action. In what follows it is intended to examine how far this is true at the present time, and to propose an alternative idea based on the disorganization of the nucleus of the cell, rather than on the separate molecular actions of radiation.

RELEVANT FEATURES OF THE BACTERIAL CELL

A cell of *Escherichia coli* is a short cylinder of radius 0.5 micron and length two microns. It contains about 0.8×10^{-14} grams of DNA and 40×10^{-14} grams of RNA (Hanawalt, 1959). The dry mass of a cell is 25×10^{-14} grams so that DNA forms about three per cent of the cell and RNA about 16 per cent of the cell. Protein forms 60 per cent, leaving 20 per cent for lipids, phospholipids, polysaccharides, amino acid pool and so on.

Evidence from the observation of tritium labeling in colonies grown from one cell indicates that the DNA is in one piece and can divide once (Forro and Wertheimer, 1959) in a manner which would be correct if the DNA were a double molecule, as is today supposed. The predominant segregation of the tritium, as revealed in radioautographs taken by Dr. F. Forro, is into two parts. On the basis of a series of such experiments, Forro has drawn the conclusion that the DNA in the cell, which is labeled by the thymidine, is capable of one equal division. The new DNA is predominantly formed, after this division, from unlabeled thymidine, and very little of the once divided label distributes itself further.

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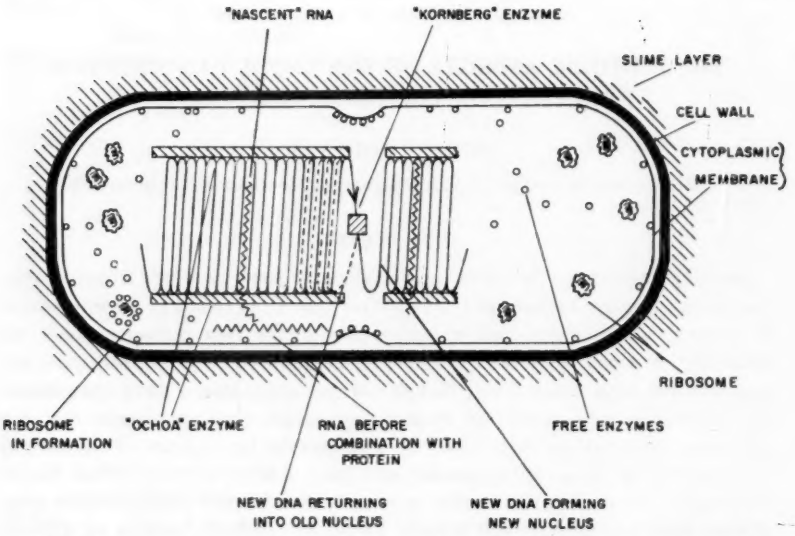


FIGURE 1. A schematic picture of a bacterial cell. The DNA is represented as in one long coil, dividing at the end. On the side of the rotating coil of DNA is an array of enzymes which are called "Ochoa enzymes" which cause the synthesis of RNA in the appropriate nucleotide sequence. The RNA emerges into the cell, coils up and combines with protein to form ribosomes. The ribosomes are the protein synthetic units (in this theoretical representation) and make protein, some of which acts as enzyme.

There is also evidence that the DNA is concentrated in a region generally referred to as a "nuclear body," but which we shall call the nucleus (Caro et al., 1958). Evidence is beginning to accrue that DNA synthesis continues, at least to a first approximation, in a uniform way (McFall and Stent, 1959; Van Tubergen, 1959). Now, in a less satisfactory way, it is accepted by many that cellular DNA somehow "makes" RNA and that the RNA combines with protein to form "microsomal particles" or better "ribosomes." The ribosomes form a whole pattern of size, as characterized in the ultracentrifuge, and also have a kinetic interrelationship which is being rapidly worked out by Roberts and the group at the Carnegie Institution of Washington (Bolton et al., 1957). It is often used as a hypothesis that these ribosomes, or some class of them, are involved in protein synthesis. Measurements made by Kempner (Pollard and Kempner, 1958) on the effect of gamma rays, deuterons and alpha particles on the uptake of several amino acids, gave target volumes of roughly the size of an RNA molecule, namely 2×10^6 molecular weight, and very long and thin. Such a molecule, coiled inside a ribosome would be expected to inactivate rather like a virus, and so the radiation evidence, which fits well with virus inactivation, can be held to uphold this idea.

In order to have something definite to think about, a schematic drawing of a bacterial cell is shown in figure 1. The idea expressed by Forro's experi-

ments is shown in the most direct way by indicating the DNA in one long coil (it might easily be an aggregate of short units attached to protein), with enzymatic synthesis of new DNA at each end. The process of manufacture of RNA is shown as taking place on the coil by a strip of "Ochoa" enzyme which forms RNA as a coil of DNA turns around in its synthetic process. The RNA is indicated as being first on the coil of DNA, where it has received its specific ordering, and then in the cytoplasm, where it presumably coils up and attracts around it the protein units which make it form into ribosomes, many of which are shown. The ribosomes act on the proper precursors, which may be amino acids, or properly activated amino acids, to form protein molecules, some of which go to form more ribosomes and some of which are cellular enzymes.

The enzymes themselves are shown on the protoplast membrane where they may function as "permeases," and in the cytoplasm. Outside the protoplast membrane is the cell wall.

SUMMARY OF RADIATION EFFECTS

Some of the radiation effects which have been studied are summarized in figure 2. As is well known, much the most sensitive effect of radiation is on cell division. It can be seen that D_{37} for the formation of colonies, which requires cell division, is around 5,000r. In TRIS buffer it is lower, being 3,000r. The growth of cell mass able to scatter light, referred to as "optical density" is less sensitive and apparently not simply logarithmic. The uptake of phosphate and sulfate are both sensitive, but less so than for division: the uptake of sulfide is still less sensitive, while the uptake of methionine is very hard to prevent.

We can mention a few more radiation effects. Measurements of the inactivation of enzymes in bacterial cells have not been carried out nearly often enough. However, for betagalactosidase Pollard and Barrett (1959) found that the amount of cobalt irradiation needed to diminish enzyme activity in cells was very nearly the same as that for the dry enzyme. Hutchinson (1960) has made somewhat similar observations on several enzymes in yeast and Powell and Pollard (1955) found very little difference for cytochrome oxidase and succinic dehydrogenase in intact cells of *B. subtilis* as compared to lysed and dried preparations. There seems to be no reason to suppose that in a cell radiation has an effect on enzymes which differs from that in a comparable medium *in vitro*.

One important process on which radiation studies have been made is the induction of an adaptive enzyme. Billen and Lichtstein (1952) have studied the formation of formic hydrogenlyase in *E. coli*, Pauly and Rajewsky (1958) have studied lysine decarboxylase in *B. cadaveris*, and some studies by Pollard and Vogler (unpubl.) on the effect of gamma rays on the induction of betagalactosidase in *E. coli* have been made. All the experiments indicate a marked effect at about 30,000r, but the actual kinetics of induction are not fully established. In the case of betagalactosidase, at the normal time for induction a little enzyme appears in cells which have received 21,000r, but

this is not followed by the usual sharp rise in enzyme production. Such a rise does occur, but is delayed by about an hour. The production of enzyme by cells already adapted seems (in three experiments) to show an increase over the control for the first hour or so, for doses up to 21,000r, but then lags behind. The respiration of *E. coli* after irradiation has been studied by Billen, Stapleton and Hollaender (1953). Doses in excess of 60,000r are needed for a marked effect to be observed.

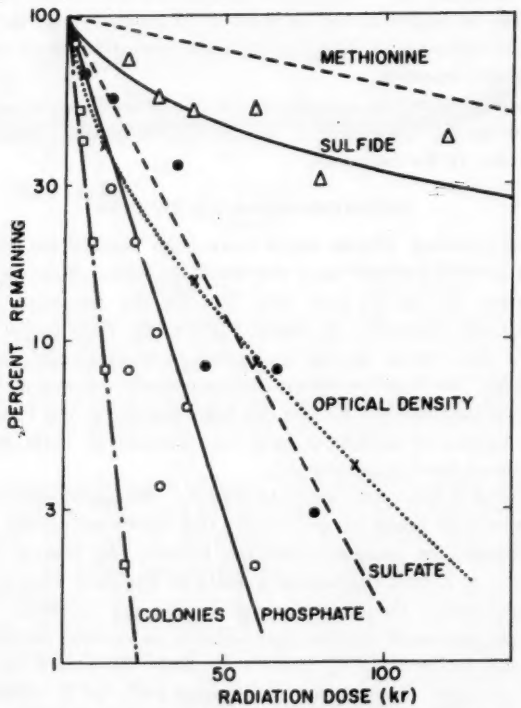


FIGURE 2. Reproduction of a graph from Pollard and Kennedy. It shows logarithmic dose response plots for colony counts, optical density, and phosphate, sulfate, sulfide and methionine uptakes.

The capacity to develop bacteriophage was studied by Pollard, Setlow and Watts (1958). It is about one-fourth as sensitive as the ability to divide, for T1 phage and one-fortieth for T2, which has a different type of DNA from the host.

Pollard and Vogler have made some measurements on the amount of DNA which gives the Keck indole reaction after irradiation. It follows very closely the reaction of the optical density as can be seen from the figures in table 1.

TABLE 1

Dose		Minutes				
		0	30	60	90	120
Control	DNA	0.11	0.12	0.14	0.22	0.26
	Optical density	0.10	0.12	0.15	0.20	0.24
	Ratio DNA/O.D.	1.1	1.0	0.95	1.1	1.1
15,000r	DNA	0.09	0.11	0.12	0.16	0.21
	Optical density	0.10	0.11	0.13	0.18	0.22
	Ratio DNA/O.D.	0.9	1.0	0.9	0.9	0.9
30,000r	DNA	0.08		0.09	0.13	0.20
	Optical density	0.10		0.13	0.16	0.18
	Ratio DNA/O.D.	0.8		0.7	0.7	1.1
60,000r	DNA	0.08	0.10	0.12	0.09	0.09
	Optical density	0.08	0.08	0.09	0.09	0.09
	Ratio DNA/O.D.	1.0	1.2	1.3	1.0	1.0

Somewhat the same can be said of the formation of lipid as determined by the fraction of the cells which have been grown on C 14 glucose subsequent to irradiation and which is soluble in alcohol and ether.

One effect which we (Kennedy and Pollard (unpublished)) have recently demonstrated in *E. coli* B is that the fraction of P32 which enters the ribosome moiety, as determined by separation on a DEAE ion exchange column, is drastically reduced for doses as low as 30,000r. The figures we obtain are shown in table 2.

A representative elution diagram is shown in figure 3.

At the same time as this reduction is observed, a fraction tentatively associated with ATP, GTP, UTP, CTP components and free phosphate, with the latter predominating, sharply *increases*. We observe no effect on the ion exchange pattern for S 35 labeled components. We observe no effect on the ultraviolet absorption pattern, on the sedimentation diagram and on the folin reacting components for doses up to 500,000r. Our results on the sedimentation diagram are in broad agreement with the experiments of Billen and Volkin (1954).

We can thus summarize the findings.

High sensitivity (D_{37} about 4,000r) for cell division.

Next highest (D_{37} about 12,000r) new ribosome and RNA formation.

Medium sensitivity, PO_4 and SO_4 uptake, adaptive enzyme formation, DNA synthesis, lipid synthesis.

TABLE 2

Dose	6,000	12,000	30,000	50,000	180,000
Per cent of normal in ribosome "A" peak (large particles)	130	50	20	0	0
Per cent of normal in "B" peak (small particles and RNA)	50	50	20	0	0

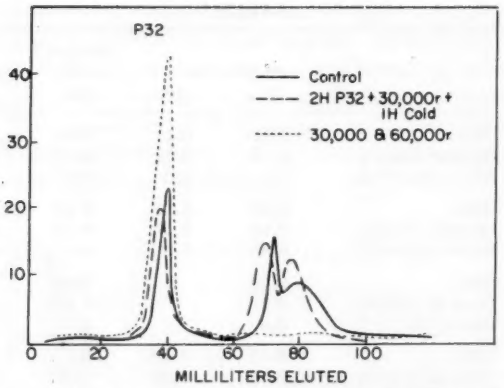


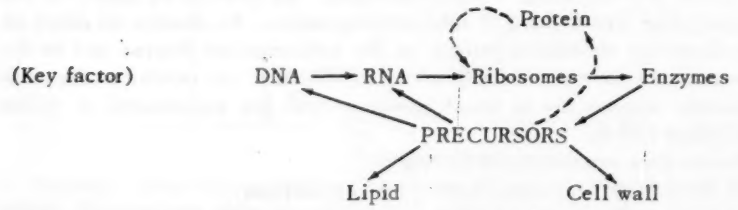
FIGURE 3. An elution diagram of pressure-opened *E. coli* cells placed on a DEAE column. The amount of P32 in each stage of elution is plotted versus the volume eluted. The full line is for unirradiated cells and the dashed line for cells labeled *prior* to irradiation. The dotted lines correspond to cells which were irradiated and then grown on P32 medium. The absence of label in the two later peaks, which correspond to ribosomes can be seen, and the increase in the earlier phosphate-nucleotide fraction. The already made ribosomes are not affected by radiation.

Low sensitivity, single amino acid uptake.

Possible *increase*, followed by diminution, production of already adapted betagalactosidase.

KINETICS OF CELL FUNCTION

Since we are dealing with a theoretical analysis, we have to use some basis for theory. The scheme suggested is as below.



The key to the cell is the steady synthesis of DNA. This is presumably enzymatic, but with the cell DNA itself as the "primer." Precursors will be needed to ensure that synthesis can go on. These precursors are certainly to a large extent mediated by enzymes. *As a first hypothesis we assume that there is always an adequate supply of the precursors.*

The steady synthesis of DNA has, as a vital "byproduct" the synthesis of RNA. As a purely speculative suggestion, the slow rotation of one whole thread of DNA could carry DNA into an "Ochoa enzyme" which was stationary and which fed the ribonucleotides into the proper configuration. The

length of RNA unit so formed would be about one turn, and there would thus be an accretion of new RNA, partly on the helical nuclear DNA and partly in the cytoplasm. If this RNA has the proper specificity, then it will, in time, encounter the appropriate protein molecules to form ribosomes and will combine with them. Possibly the specificity of the RNA involves some kind of coiling into a spring-like form, around which the protein can form itself. In any event, we need to state in our theory that free RNA becomes bound into ribosomes and that this means we have a rate of formation of RNA which is proportional to the amount of DNA present, less the rate of combination with protein.

Thus if we let D represent DNA, R represent specific RNA, E represent specific protein, we can set

$$\frac{dD}{dt} = D_1, \text{ where } D_1 \text{ is a constant}$$

$$\frac{dR}{dt} = aD - bE$$

The second term bE represents a sum of the combination of RNA + protein *and* the degradation of RNA by RNA-ase, shown by Bolton et al. (1957) to be present in ribosomes. It is ineffective on specific RNA, but might well be highly active on degraded RNA. Thus b would be expected to *increase* even if the RNA could not combine with protein.

The rate of formation of *ribosomes* is related to the amount of RNA and to the amount of protein, so we have

$$\frac{dR_s}{dt} = cRE$$

where R_s is the amount of ribosomes present.

We next have, for the synthesis of enzyme

$$\frac{dE}{dt} = gR_s .$$

This expresses the idea that the synthesis of protein, in the presence of an excess of all the precursors, is dependent only on the number of ribosomes. However, the actual increase in the amount of protein will not be so great, since the formation of ribosomes themselves will diminish the total free protein formed. Thus the equation for protein increase must be modified to become

$$\frac{dE}{dt} = gR_s - \frac{f dR_s}{dt}$$

$$= gR_s - fcRE$$

Terms in lipid synthesis presumably depend on the amount of E present, again with an excess of precursor, so we have a fifth equation for lipid, L,

$$\frac{dL}{dt} = hE$$

The five equations of cellular synthesis are then:

(1) $\frac{dD}{dt} = D_1$ or $D = D_o + D_1 t$, where D_o is an initial amount of DNA.

(2) $\frac{dR}{dt} = aD - bE$

(3) $\frac{dR_s}{dt} = cRE$

(4) $\frac{dE}{dt} = gR_s - fcRE$

(5) $\frac{dL}{dt} = hE$

It is clear, at a glance, that these equations, which are certainly oversimplified to some extent, will be hard to solve without extensive use of computers. To see some of their properties we can imagine the state briefly after cell division, when we can suppose that a quota R_s^o of ribosomes has come along with the division process. Then we can ignore fcRE compared to gR_s and we get, for *initial* starting solutions

$$E = gR_s^o t + E_o \qquad (E_o \text{ is an initial amount of protein})$$

$$R = R_o + (aD_o - E_o)t + (aD_1 - bgR_s^o)\frac{t^2}{2}$$

$$R_s = R_s^o + c[R_oE_o + E_o(aD_o - E_o) + gR_oR_s^o]t + \left[\frac{E_o}{2}(aD_1 - bgR_s^o) + (aD_o - E_o)gR_s^o\right]\frac{t^2}{2} + gR_s^o(aD_1 - bgR_s^o)\frac{t^3}{6}$$

$$L = L_o + E_o t + gR_s^o\frac{t^2}{2} \qquad (L_o \text{ is an initial amount of lipid})$$

We can comment on two features of these equations. The first is the term in

$$aD_1 - bgR_s^o .$$

It controls, in a vital way, the terms in higher powers of the time. That is, the presence of a very rapidly advancing value of either R, or R_s, as time advances, depends upon it. The two terms represent, first the rate of increase of DNA together with its ability to form RNA, which last is the interpretation one may put on the factor "a." The second, and negative term, is related to the protein synthetic power of the cell, plus the ability of a fraction of that protein to digest and so remove RNA. Unless the whole term is positive, the rapid advance with time will not occur.

The second term of great significance is

$$aD_o - E_o$$

which represents the initial potential of the cell to produce RNA, less the ability to combine with, or destroy, RNA *initially.*

We can see how the equations will produce solutions by trying the method of successive approximations. To do this we need, successively, to replace R_s^o and E_o by the new values appropriate to the solution after a short initial time. It is clear that both the terms we have just discussed will *diminish*, so that the rate of increase of R_s, R and E will not reflect the initial solutions, but will tend to have terms which are less dramatic at high values of t. It would be expected that the relative rates of increase of protein and lipid could very well be the determining factor in cell division.

EFFECT OF IONIZING RADIATION

It is natural to seek the consequences of ionizing radiation in those aspects of the cell which are most sensitive. These are cell division and the synthesis of ribosomes, with the possible addition of the synthesis of specific RNA, which may well be the determining factor in the failure of the cell to assemble ribosomes.

Cell division, as of 1959, seems to involve a set of complex factors and so it might be wise, for the present, to treat it as secondary. However, the process of synthesis of ribosomes is contained in one of our five equations of cellular synthesis. *We make, then, the suggestion that for doses in excess of 30,000 r, the quantity "c" in the third equation becomes close to zero.* We hold in reserve the added idea that "a" is lessened in value.

After irradiation, then, the appropriate equations are:

(1) $$D = D_o + D_1 t$$

(2) $$\frac{dR}{dt} = aD - bE$$

(3) $$\frac{dR_s}{dt} = 0$$

(4) $$\frac{dE}{dt} = gR_s^o \quad \text{or} \quad E = E_o + gR_s^o t$$

(5) $$\frac{dL}{dt} = hE$$

The fourth and fifth of these can be immediately integrated to yield

$$E = E_o + gR_s^o t$$

$$L = L_o + hE_o t + hgR_s^o t^2/2$$

where L_o is an initial amount of lipid and, as before, R_s^o represents the initial amount of ribosomes present.

Both these seem to be sensible. The linear increase in sulfur uptake, which would be representative of the increase of protein, here denoted by E, is immediately apparent. The increase in L should be more rapid than linear, a feature which has not been looked for carefully. However, since we know that only 20 per cent of the protein is tied up in ribosomes, it is likely that the term in $hgR_s^\circ t^2/2$ will not predominate over the linear increase until quite long times. However, the fact that lipid increase is at a more rapid rate than protein increase, as time goes on, gives a very natural explanation for the failure of cell division. Thus we can understand the formation of "snakes" for bacteria and possibly of giant cells in tissue culture studies.

Thus we can account for both the linear protein synthesis *and* the inhibition of cell division simply as a consequence of the discovery that $c = 0$.

We can now turn to the equation in R. This becomes

$$\frac{dR}{dt} = (aD_o - bE_o) + (aD_1 - bgR_s^\circ)t .$$

Integrating it gives

$$R = R_o + (aD_o - bE_o)t + (aD_1 - bgR_s^\circ)\frac{t^2}{2}$$

where R_o is the initial amount of RNA present. Obviously the numerical solution depends on the two terms involving the time. We have already mentioned that the value of b would be expected to increase, not so much as a result of radiation, as of the fact that free RNA-ase can develop and remove the non-specific RNA, which does not form ribosomes. Thus we can rather confidently suggest that the term linear in the time is zero, or nearly so, if indeed not negative, if radiation has been very effective. The term in t^2 is more open to thought. If the *new* DNA, which is what is really represented by D_1, is able to make even a fraction of specific RNA, then there is a possibility that, as time goes on, the very small contribution of this term may become significant. Thus we draw the highly interesting conclusion, that a cell in which the combination of protein and RNA to form ribosomes has been reduced by the formation of non-specific RNA may gradually recover the ability to make specific RNA, if the *new* DNA has potency. Thus after a long wait, the term in t^2 begins to have an effect; the specific RNA begins to grow, ribosomes begin to form, and the cell may then, after a long "depressed" existence, return to its normal rates.

When this occurs, the outstripping of protein by lipid will be modified, and the cell may accordingly divide. Just this process was observed by Deering (1958). Those cells which had received relatively small amounts of radiation and formed snakes, had the power, on occasion, to "bud off" a normal cell, which then subsequently divided in a typical way.

SUGGESTIONS FOR THE MECHANISM OF RADIATION ACTION

Since the work of Hutchinson (1960) and to a lesser extent of Powell and Pollard (1955) indicates that diffusible agents add a distance of only 30 Å

to the range of action of local ionizations, and since the effects described correspond to rather large "targets," it is not unwise to try to make some kind of a radiation statistical analysis in order to set what kind of mechanism might be at work.

If we take the D_{37} for the inhibition of ribosome formation to be 12,000r, then the target volume, assuming a single primary ionization to be involved, is 1.65×10^{-16} cc. The volume of the nucleus of the cell, treated as a sphere of 2500 Å radius is 6.5×10^{-14} cc. This is four hundred times too large. The volume of the DNA in the cell is 6.2×10^{-15} cc which is forty times too large. If we were to suppose that there is a nuclear membrane, and that the thickness Δx were to be determined by the target volume, we then have, for a nucleus of radius 2500 Å, or 2.5×10^{-5} cm the relation

$$4\pi \times (2.5 \times 10^{-5})^2 \Delta x = 1.65 \times 10^{-16}$$

$$7.8 \times 10^{-9} \Delta x = 1.65 \times 10^{-16}$$

$$\Delta x = 2.2 \text{ Å}$$

This hardly seems to be plausible: it is too thin. However, if we assumed that the unit in the cell, which we have designated the "Ochoa" enzyme, which forms RNA while the specific DNA rotates under it, has a length of 5000 Å, the assumed length of the nucleus, it could be represented as a cylinder of radius r and we can then set

$$\pi r^2 \times 5 \times 10^{-5} = 1.65 \times 10^{-16}$$

$$r = 103 \text{ Å}$$

Such a radius is too thick for most normal protein molecules, but it is much closer to a real physical object than we have yet attained. Thus we might suggest that the process of bringing DNA into a synthetic region involves the presence of an organized set of enzyme molecules arranged in some kind of sequence as a physical object. These can be broken apart by the action of an ionization in, or near, their entire volume. Such a breakage might not destroy either the production of DNA or of RNA, but it might well destroy the production of specific RNA and hence of ribosomes. Thus we find the best suggestion that can be made is that ionizing radiation acts to destroy the specific integrity of a part of the nucleus, possibly a part of the membrane, which is used to transfer the specific base relationships of DNA to RNA molecules. The specific object has a volume of about 1.6×10^{-16} cc, less than the nucleus, the cellular DNA and even the whole nuclear membrane. It is thus a part only of the nuclear structure.

Since the disruption of this mechanism is supposed either to stop synthesis of RNA, or alternatively to make RNA which is vulnerable to RNA-ase, there will be a stoppage in the formation of ribosomes, without the action of those already existing being stopped. Thus the manufacture of protein will continue at a definite rate, and since enzymatic action is related to protein, any cellular function which requires enzymes will continue at the same rate.

In figure 4 is shown uptake of $S350_4$ and $P320_4$ in normal and irradiated cells in the same culture as measured by Pollard and Vogler. The counts for P32 and S35 were separated by using a foil of sufficient thickness to stop the S35, while reducing the P32 only by 20 per cent. The normal curves were adjusted to fit at 60 minutes for both isotopes. It can be seen that in the irradiated cultures a period of linear uptake, *at the same rate in both cases*, takes place after an initial time. This is presumably due to protein formation from non-increasing ribosomes, which is directly measured by S35 and indirectly measured as enzyme action for P32. This enzyme action presumably acts simply to incorporate the P32 in a form which will remain in the cells; no further specificity is required of it.

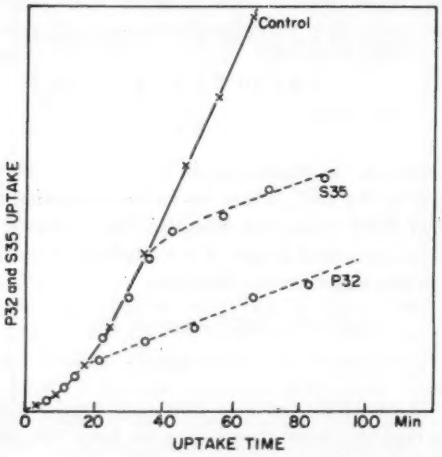


FIGURE 4. The uptake of P32 and S35 in the same culture of *E. coli* after 36,000r as observed by Pollard and Vogler. The linear portion has the same increase of protein, directly measured for S35 and indirectly as enzymic action for P32.

We can turn to one or two other aspects of radiation action on *E. coli* cells. Since the formation of protein, E, continues in a linear way, the respiration of the cell should also continue, only showing a diminution as the number of cells in a culture falls below that of an exponentially increasing culture. This was found by Billen, Stapleton and Hollaender (1953). There should be an increase in some enzymes, namely those which would be "tied up" in ribosomes, but which can no longer be held by specific RNA. In particular, a definite increase in RNA-ase is predicted.

The uptake of a single amino acid is presumably due, in part, to the functioning ribosomes. These are not *reduced* in number by relatively small doses and so the uptake of methionine, proline, etc., should require much larger doses before an effect is shown. This explains the observations of Kempner, Morowitz, Hutchinson and the author. Only when the radiation

dose has definitely begun to inactivate the individual ribosomes, namely at about 100,000r, would an effect be expected. This is as found.

The formation of an adaptive enzyme does not readily fall into this scheme. At doses of 21,000r adaption does occur, though with unusual and delayed kinetics. It may be that the term in D_1, the new DNA, is involved in the formation of a new protein and that so long as D_1 is not reduced to zero, an adaptive enzyme can be formed. Since betagalactosidase has a large molecular weight (Pollard and Barrett, 1959), it is possible that the new enzyme is formed by the aggregation of enzymes already capable of being made, in which case the old ribosomes could perhaps be sufficient for the new task.

SUMMARY

A series of equations for cellular synthesis based on the steady formation of DNA, followed by successive formation of RNA, "ribosomes," protein and lipid are developed.

Using the observation that radiation inhibits the formation of ribosomes, the simplified equations are solved. The linear uptake of SO_4 and PO_4 is explained and a suggestion for the formation of long filaments, or giant cells is made. The equations contain the possibility that a delayed budding-off of a new cell can occur.

The hypothesis is presented that the disruption of a part of the nucleus, namely the organized set of enzymes which "make" RNA, is responsible for the action.

Consideration is given to enzyme formation, which can conceivably increase, and, in particular, it is predicted that RNA-ase should increase after irradiation.

The uptake of amino acids, the process of respiration and of adaptive enzyme formation are discussed.

ACKNOWLEDGMENTS

The author wishes to acknowledge the sustained impetus given by his whole group, in particular Drs. Setlow, Hutchinson, Morowitz, Woese and Kempner. The importance of the integrity of the nucleus was pointed out to him by Dr. R. B. Roberts and thanks are due to him and to Drs. Bolton and Britten of the Carnegie Institution for much stimulation and understanding.

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RADIATION STUDIES ON THE NATURE OF CHROMOSOME BREAKAGE*

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When biological materials are irradiated, one of the things that is readily noticed is that the radiation can break the chromosomes in the nucleus of the cell. In the past this phenomenon has played a rather large role in the development of theoretical radiobiology. As a matter of fact, if we look into the classic book on radiobiology and target theory, Lea's book "Action of Radiations on Living Cells" (1945), we find that approximately a third of the book is concerned with chromosome aberrations.

The study of these aberrations was invaluable in the development of the target theory, which of course explains radiation effects in terms of simple direct action of the radiation on specific loci, or molecules, within the cell. If the dose action curves for such effects of radiation as cell death or delay of cell division were studied, it could only be inferred that targets of certain number and size were being hit and inactivated. However, the chromosomes provided gigantic "molecules" of known shape that could easily be seen with an ordinary compound microscope. Here, before the eyes, were the targets themselves. We were no longer removed by one or more steps from the initial damage. There were certain aberrations such as simple terminal deletions that were obviously brought about by one break of the chromosome, and other aberrations that were the obvious result of the interaction of two or more breaks. The one-break aberrations increased linearly with the dose of x-rays, indicating that these were one-hit phenomena, that is, that the breaks were produced with a certain probability (p) directly proportional to the dose of radiation. The two-break aberrations increased, as we would expect on the basis of probability, as the square of the x-ray dose. This, because if one break is produced with a probability (p) the probability of two breaks being there at the same time and essentially in the same place would be $p \times p$ which would be proportional to the square of the dose. Furthermore, the breaks in the cell occurred at random; that is, if the number of cells having zero, one, two, three or more breaks were examined, they were found to fit the Poisson distribution. Here then were all the elements of the target theory, only we no longer had to make inferences from the numbers of survivors; that is, we no longer had to base our conclusion on those cells that were not hit but could now study directly the damage induced.

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By using this system, other biophysical studies could also be made. For instance, calculations were made concerning the numbers of ionizations necessary to break a chromosome. Calculations were made about the distance apart that two broken chromosome ends can be and still interact with one another to form a two-break exchange and also calculations were made about the efficiency of radiations of different quality.

In another field, too, the study of chromosome aberrations has made great advances. This is genetics, where radiation not only can provide new mutations for practical applied biology but also can give us the opportunity to study the mutation process itself.

The mutations induced by radiation can be conveniently divided into two classes: the intragenic mutations or true point mutations that are changes within the gene itself, and the intergenic mutations that are the result of changes within and between the chromosomes. This latter class of mutations is the result of the same two processes, breakage and the subsequent rejoining of the breaks in new combinations, that result in chromosome aberrations. As a matter of fact, the aberrations are only a subclass of the larger group of intergenic mutations.

Today, in our laboratory when we study the effects of radiation in inducing chromosome aberrations, we observe the same final event, altered chromosomes, but we are asking different questions from those of a few years ago. Our main questions today deal not only with biophysics but also with the chemical nature of the genetic changes and the metabolic repair of these changes. These problems have been attacked by methods that can be classified as theoretical radiobiology.

For instance, since the intergenic mutations or the aberrations observed are the result of both breakage and the subsequent rejoining of the breaks, it is important for any understanding of the underlying phenomena to be able to separate these two processes. We have done this by relying on fractionated doses of radiation with treatments between the doses. The rationale behind this is that with the first dose chromosome breaks will be produced. If a second dose is administered before the first breaks rejoin, the breaks from the two doses will be present in the cell concurrently and able to rejoin with one another. In this case the yield of two-break or two-hit aberrations will be the same as if the dose were not fractionated and proportional to the square of the total dose. If, however, enough time elapses between the two doses so that the first group of breaks can rejoin before the second group is induced, then the yield is simply the sum of the two-hit aberrations induced by each of the doses.

If we give the first dose of radiation, and thus put in all the breaks from this dose and then immediately after treat with various specific metabolic inhibitors, we can see whether these inhibitors keep the breaks open long enough to rejoin with breaks from a second dose. Thus we have a quantitative measure of the processes involved in the rejoining (or repair) of the chromosome breaks separated from the breakage phenomenon itself.

By just such studies, we found that the rejoining of breaks was inhibited by all the various inhibitors of cellular respiration and of ATP formation (Wolff and Luippold, 1955); that the rejoining was not a passive process as we had tacitly believed previously but was an active metabolic event that required energy. Because these breaks could stay open for relatively long periods of time, up to three hours, depending on the dose of radiation, and because they required energy to rejoin, we thought that the chemical bonds formed when breaks rejoin were not ionic bonds, or hydrogen bonds, or any bonds that required only electrical factors for their rejoining, for these should rejoin very, very rapidly and not require an external source of energy. On the contrary, we feel that the bonds formed are strong covalent bonds.

In an attempt to define these links and also to help understand just how a chromosome is constructed, we decided to apply specific protein synthesis inhibitors between two doses to see if this would keep breaks open (Wolff, 1959). Protein synthesis inhibitors were selected, of course, because the chromosomes consist of nucleoproteins, and we might therefore expect protein synthesis to be involved in the rejoining of the breaks.

EFFECTS OF INHIBITORS OF PROTEIN SYNTHESIS

The actual experiments consisted of administering 600r *in vacuo* to *Vicia faba* seeds that had been soaked for 18 hours. This would produce breaks that remained open for about 30 minutes before rejoining. After at least 75 minutes, a second dose of 300r was given to the same seeds. The seeds were then allowed to complete germination; and two days later, when the first cell divisions were occurring in the root tips, the slides were made and 300 metaphase cells were scored for two-break aberrations. In table 1 it can be seen that the application of 300 μg/ml of chloramphenicol, a known inhibitor of protein synthesis, between two doses of radiation prevents the breaks from rejoining within 30 minutes and keeps them open for at least 75 minutes. It may be seen that the treatment with chloramphenicol after a dose of 600r *in vacuo* does not affect the aberration yield. It is the same as if water was applied. Nor does chloramphenicol affect the aberration yield when given before a dose of 300r. However, when chloramphenicol is present between the two doses, then the breaks from the first dose remain open for at least 75 minutes and capable of forming exchanges with breaks from the second dose. The aberration yield is then 0.373 exchanges per cell and is proportional to the square of the total dose. If water was present between the two doses, then instead of the breaks staying open, they rejoined before the second dose and the aberration yield was nearly the sum of the yield of the two doses. The control value, however, is somewhat higher than this sum, indicating that even in the controls a few of the breaks do not rejoin in the 75 minutes. In the presence of chloramphenicol, however, *all* the breaks do not rejoin and the theoretically expected value is obtained. It therefore looks as though chloramphenicol (or protein synthesis inhibition) can keep breaks open.

TABLE 1

The effect of protein synthesis inhibitors on chromosome break rejoining

Dose 1 (r; *in vacuo*)	Interval		Dose 2 (r)	Two-hit aberration yield per 100 cells observed	Expected aberration yield	
	Time	Treatment			With rejoining	Without rejoining
600	75 min.	H_2O	...	10.0 ± 1.8		
600	75 min.	Chloram.	...	9.3 ± 1.8		
...	75 min.	H_2O	300	9.7 ± 1.9		
...	75 min.	Chloram.	300	10.3 ± 1.9		
600	75 min.	H_2O	300	25.9 ± 2.4	19.7	39.3
600	75 min.	Chloram.	300	37.3 ± 3.6	19.7	39.3
600	110 min.	H_2O	...	13.0 ± 2.5		
600	110 min.	Aureo.	...	14.0 ± 2.4		
...	110 min.	H_2O	300	10.4 ± 2.0		
...	110 min.	Aureo.	300	13.6 ± 2.3		
600	110 min.	H_2O	300	30.7 ± 4.5	23.4	48.0
600	110 min.	Aureo.	300	51.5 ± 5.1	27.6	55.2
600	90 min.	H_2O	...	18.5 ± 3.0		
600	90 min.	Pen.	...	15.2 ± 2.5		
...	90 min.	H_2O	300	12.3 ± 2.0		
...	90 min.	Pen.	300	11.3 ± 1.9		
600	90 min.	H_2O	300	31.6 ± 3.7	30.8	62.0
600	90 min.	Pen.	300	28.9 ± 3.5	26.5	52.7

All chemical concentration equalled 300 μg/ml.
Chloram. = Chloramphenicol; Aureo. = Aureomycin; Pen. = Penicillin.
Data from S. Wolff (1959).

However, it should be pointed out that just because chloramphenicol inhibits protein synthesis in bacteria, it does not necessarily mean that chloramphenicol does the same thing in beans. To be certain that protein synthesis was involved, we decided to try out other known protein synthesis inhibitors, such as aureomycin. In table 1 we also see that the application of 300 μg/ml of aureomycin after a dose of 600r *in vacuo* has no effect on the aberration yield. Nor does the application of aureomycin have any influence on the aberration yield when given before a dose of 300r in air. However, when aureomycin is present between the two doses of radiation, again the breaks from the first dose cannot rejoin but stay open and are capable of forming exchanges with breaks induced by the second dose 110 minutes later. Again, if water was present between the two doses, the aberration yield was nearly the sum of the aberration yield induced by the two doses. In order to make certain that the effect of these two inhibitors of protein synthesis was not due to some unknown antibiotic effect that both might have since both are antibiotics, we performed the same experiment using another antibiotic, penicillin, which is not a protein synthesis inhibitor. In table 1 we can see that penicillin has no effect either after or before a dose of radiation nor does it have an effect between two doses of radiation. In this case the aberration yield is simply the sum of the yield of the two half-doses, indicating that in the presence of penicillin, as well as in

the presence of water, the breaks from the first dose rejoin before the second dose is given.

TESTS OF PROTEIN INHIBITION

It was necessary at this stage of the experiments to establish that the inhibitors we were using did inhibit protein synthesis in the bean. Chloramphenicol has been reported to be a specific protein synthesis inhibitor in bacteria when used at 30 μg per milliliter (Gale and Folkes, 1953). In these experiments we were using a higher organism and a higher concentration of the inhibitor. We decided, therefore, to treat soaked beans with C^{14}-labeled glycine and then to extract the proteins from these beans and check for the incorporation of the radioactive glycine into the protein. We have performed three separate experiments (data from G. D. Novelli and S. Wolff, unpublished). In the first experiment we found that there was a 13.3 per cent inhibition of protein synthesis when the beans were treated with 300 μg/ml of chloramphenicol. In the second experiment we observed a 33.2 per cent inhibition, and in the third experiment we observed a 24.7 per cent inhibition; and so it looks as though chloramphenicol at these concentrations in the beans can inhibit incorporation of labeled amino acids into proteins.

To see whether this was a specific inhibition, we decided to check the effect of chloramphenicol on the incorporation of P^{32} into the nucleic acid of Vicia. Again the soaked beans were incubated for three hours, this time in $H_3P^{32}O_4$, and then the nucleic acids were extracted from the root tips. The radioactivity was then checked in these extracted nucleic acids. In table 2 it can be seen that whether or not chloramphenicol is present the specific activity registered in counts per milligram per second is the same, indicating that chloramphenicol does not inhibit the incorporation of P^{32} into nucleic acids of Vicia.

In an attempt to see whether deoxyribonucleic acid (DNA), which of course is a major constituent of chromosomes, was being inhibited or affected by chloramphenicol, we incubated some other beans in tritiated thymidine. Then squash preparations and autoradiographs were made. In no cases at the particular time when we ordinarily would irradiate and find breaks rejoining did we get any incorporation of the tritiated thymidine into

TABLE 2

Incorporation of P^{32} ($H_3P^{32}O_4$) into nucleic acids of Vicia

Experiment	μg/ml Chloramphenicol	Cts/mg/sec
I	0	46
	300	54
II	0	79
	300	81
III	0	99
	300	106
Background		0.181 cts/sec

the nuclei of the cells, indicating that there is no synthesis of DNA going on at the time that breaks rejoin. Actually other experiments by Davidson (unpublished) have shown that the incorporation of tritiated thymidine does not occur until the seeds have been germinating for 36-48 hours. This is at least twice as long as is necessary to observe the rejoining of breaks. Thus it is highly improbable that when a break rejoins, DNA synthesis causes the rejoining. The results of these experiments, so far, have indicated that inhibitors of protein synthesis prevent the rejoining of chromosome breaks, and from this it follows that protein synthesis is necessary for breaks to rejoin. The reagents that do keep these breaks open have been found *biochemically* to inhibit protein synthesis but *not* to affect nucleic acid synthesis in the beans at the time when the experiments are being performed. In figure 1 we can see a possible interpretation for these

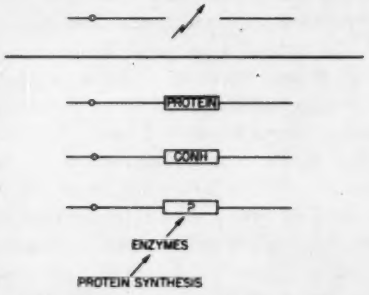

FIGURE 1. Possible ways in which protein synthesis can cause rejoining of chromosome breaks. The three lower lines represent, from above, synthesis of a protein, synthesis of a peptide bond, synthesis of an unknown substance.

experiments. Here we find represented a break in the chromosome. In order for this break to be repaired, protein synthesis has to occur. It might be that a block of protein is built when the breaks rejoin or it might be that a peptide link is synthesized. However, at least a third possibility does exist and this is that some unknown product necessary for the breaks to rejoin is made and that the synthesis of this product is dependent upon, let us say, formation of enzymes, which of course are protein. Therefore, by blocking protein synthesis we are affecting the actual rejoining of the breaks only indirectly and are still one or more steps removed from defining the bond formed at the time rejoining occurs. However, I think that our present experiments tend to rule out the idea that this piece of unknown substance would be DNA. Since the gene is considered to be DNA, the fact that DNA is not synthesized when breaks rejoin to give these aberrations or mutations helps to strengthen the terminology whereby these are known as intergenic alterations, for it appears that the genic DNA is not itself involved in these breaks (Sparrow et al., 1952).

Now all that has been said about these aberrations and the metabolic stimulation of the repair of breaks in addition to giving us some clues as to

the organization and structure of the chromosome also tells us about the possible methods of affecting the production of intergenic mutations such as translocations and inversions. Beyond this, we may only speculate. However, recent work has brought to light genetic phenomena, as yet uncharacterized as to whether or not they are intragenic or intergenic, that can be correlated with, and possibly explained by, the effects observed on aberrations. For instance, Sermonti and Morpurgo (1959), working with the somatic segregation of mutants induced in Penicillium with nitrogen mustards or x-rays, found that this segregation and presumably the numbers of mutations induced is decreased by post-treatments with about one per cent $MnCl_2$. This reagent, $MnCl_2$, has been found by Ernster and Low (1955) to stimulate ATP synthesis in mitochondria. Since we had found that in-

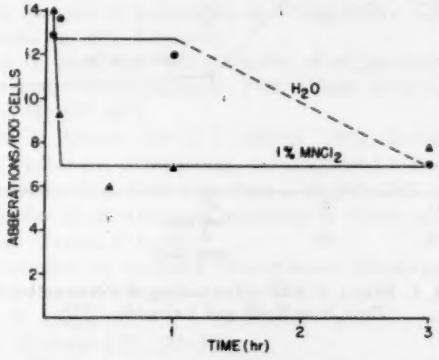

FIGURE 2. The effect of $MnCl_2$ on the rejoining of chromosome breaks.

hibition of ATP prevented chromosome breaks from rejoining and conversely that the addition of exogenous ATP stimulated repair, Sermonti and Morpurgo (1959) have postulated that their effect is on the repair of genetic damage. In figure 2 may be seen the results of experiments to test directly whether $MnCl_2$ can stimulate the rejoining or repair of radiation induced chromosome breaks in Vicia. The experiments consisted in treating the beans with one per cent $MnCl_2$ and then irradiating at different intensities. As the intensity of the radiation decreases, some of the breaks formed by the first part of the radiation will restitute before the last of the breaks are produced. Therefore, at low intensity there will be fewer breaks in the cell concurrently than if all the breaks were put in at once with high intensity radiation. The length of time that the breaks remain open is reflected as the length of time over which there is no decrease in two-hit aberration yield as intensity decreases. It may be seen that if the 600r is given in water the breaks remain open for at least one hour and that they then close sometime before three hours. Actually, other experiments have shown that they really remain open for at least two hours (Wolff, 1954). However, when $MnCl_2$ is added to the beans for one-half hour before irradia-

tion and they are then irradiated in the solution, there is no difference at the very highest intensity used, but by six minutes the breaks are already restituting and the two-hit aberration yield decreasing. For comparison's sake, we can see in figure 3 that the addition of ATP yields a qualitatively similar picture (Wolff and Luippold, 1956). Thus it is seen that the explanation of Sermonti for his genetic effect is consistent with what these chemicals can do to affect the repair of chromosome breaks.

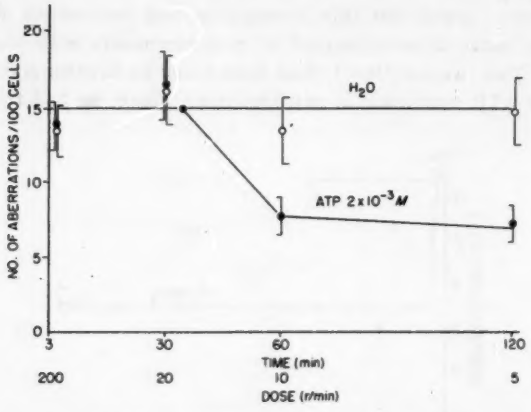

FIGURE 3. Effect of ATP on rejoining of chromosome breaks. Data from Wolff and Luippold, 1956.

Similar indications that postirradiation metabolism can modify the radiation-induced genetic damage can also be seen in the work of R. F. Kimball (Kimball et al., 1959a, 1959b). Kimball finds in *Paramecium aurelia* that the numbers of mutations induced can be decreased by keeping the animals from dividing. This seems to allow a repair of the initial damage before it becomes a final mutation. In animals that are metabolizing rapidly, the decrease occurs rapidly, again implicating a metabolic repair of a premutational damage.

SUMMARY

We have been able to study separately the phenomena of chromosome breakage and rejoining, phenomena that are involved in the production of intergenic mutations. It seems as though protein synthesis is necessary for the rejoining to occur and so far our results are not inconsistent with the interpretation that the actual bonds formed are protein links. We do know that RNA and DNA synthesis do not seem to be involved. However, it still might be that the protein synthesis is only making enzymes that are necessary for the repair of these breaks and that we have not as yet characterized the bond formed. It has also been established that cellular metabolism is important in the genetic damage repair that is manifested as rejoining of

breaks. It has been found that a similar postirradiation treatment can decrease, in other organisms, genetic damage that may be point mutations.

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THE ACTION OF RADIATION ON MAMMALIAN CELLS*

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Intensive study of the radiobiology of the mammalian cell is necessary in order to achieve more secure understanding of many facets of the mammalian radiation syndrome, and to supplement other kinds of studies on the long-term genetic effects of radiation in man. But aside from these important applications, the response to radiation of the mammalian cell considered as an independent organism, is a fascinating subject for investigation. With the development of tools that permit long-term mammalian cell growth with constancy of karyotype (Puck et al., 1958), quantitative plating of single cells to grow into discrete colonies, and the ability to detect, and isolate rare mutants (Puck et al., 1956), almost all of the classical studies on the irradiation of independent microorganisms like *E. coli* have become applicable to normal and malignant mammalian cells *in vitro*. In addition, the ability to set up a stable culture of the cells from any individual, the precision with which the chromosomes may be visualized and identified (Tjio and Puck, 1958a, b) and the possibility that cells may be manipulated *in vitro* and then returned to *in vivo* situations for further study make available to these studies operations which have not generally been possible with the classical microorganisms.

I. SINGLE CELL SURVIVAL CURVES[1]

Single cell survival curves permit accurate assessment of the dose needed to destroy the reproductive capacity of the individual cell. It was demonstrated that the mean lethal dose for normal diploid human cells actually is approximately 50r, while that of various hyperploid, aneuploid cells is two or three times larger (Puck and Marcus, 1956; Puck et al., 1957).

Typical survival curves of different human cells are presented in figure 1. These demonstrate that the aneuploid hyperploid cells, regardless of

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[1]Throughout this paper, radiation dose will be expressed as roentgens—that is, as measured by placing an r-meter in air at the site of the sample to be irradiated. Conversion to rads which would permit absolute calculation of the number of ionizations produced would in some cases require a correction factor, perhaps as high as 1.40 where cells have been plated on glass, as has been suggested (Spiers, 1949; Wilson, 1950, 1958). The use of "lethal" and "killing" and similar expressions in this paper when applied to single cells always refers to destruction of the capacity to multiply indefinitely and form a macroscopic colony.

their origin in normal or cancerous tissue, display a multiple hit curve, with a hit number close to two, while normal diploid human cells have a hit number much closer to one. Also, the slope of the linear portion of the curves for the hyperploid cells is only about half that for the diploid cell, indicating a greater radioresistance of the cells with supplementary chromosomes. As a measure of radioresistance of the sensitive sites within the cell, the mean lethal dose, D^o, will be here employed, which is the dose in roentgens needed to reduce the survivors to 37 per cent of their former level, as computed from the straight line portion of the curve.

These data are concerned only with events which destroy the cell's ability to reproduce without limit, as measured by the ability of single cells to form colonies capable of unlimited multiplication. Any other effects of the radiation are not measured in these particular experiments. These experiments reveal that the amount of energy needed to kill mammalian cells reproductively is exceedingly small—the mean lethal dose, D^o, representing an energy absorption equivalent to a temperature rise of only 10^{-4} degrees (Puck and Marcus, 1956; Puck et al., 1957). Hence, it may be concluded that the lethal effect can be initiated in structures whose collective dimensions are huge on a molecular scale. Moreover, since irradiated cells which have lost the power of sustained reproduction, can carry out active metabolism including many kinds of specific macromolecular biosynthesis for days or weeks (Puck and Marcus, 1956; Puck et al., 1957; Marcus and Tolmach, 1958) the damage responsible for reproductive failure apparently is not reparable by the cell. Finally, the low hit numbers of the survival curves demonstrate that the critical structures do not exist in tens or hundreds of extra replicates which can continue the critical function even though one or two units have been inactivated.

These considerations strongly suggest that the genetic structure of the cell is the locus for the critical events which destroy reproductive function while permitting many physiological activities to continue. The cell chromosomes are of a size which would admirably account for the magnitude of the mean lethal dose. These structures occur either uniquely or in very small replicate numbers inside each cell. Many, if not most of the genes carried on the chromosomes are essential for reproduction. Finally, while all other cell structures presumably are ultimately synthesized by means of the fundamental coded information carried on the chromosomes by the genes, the code-carrying mechanism itself cannot be replaced by any other structure if it is lost or destroyed. A variety of experimental evidence (Puck, 1959a) including the demonstration of chromosomal and metabolic mutants among the radiation survivors, and scoring of various types of chromosomal aberrations, has demonstrated that in human cells, x-irradiation produces chromosomal damage in an amount easily sufficient to account for the loss of reproductive power among the cell population.

It remains to explain the different shapes of the survival curves exhibited in figure 1. At least three types of genetic damage leading to cell death can be anticipated (Puck, 1959b). The first is death by a single-hit process, in which a single ionization is sufficient provided it falls within an

appropriate region inside the cell. Examples of such a lethal effect would be mutation of a single vital gene in a haploid cell, or inactivation as a result of a single radiation event of any other unique and critical structure in a polyploid cell. The second is a multiple hit, specific process, in which reproductive death requires hits at a number of specific sites, the simplest example of this being a polyploid cell in which death may require inactivation of any vital gene in all the copies in which it occurs. Finally, reproductive death can occur from interaction of multiple, but non-specific chromosomal lesions, as when dicentric bridges form through union of fragments produced from breakage of different chromosomes.

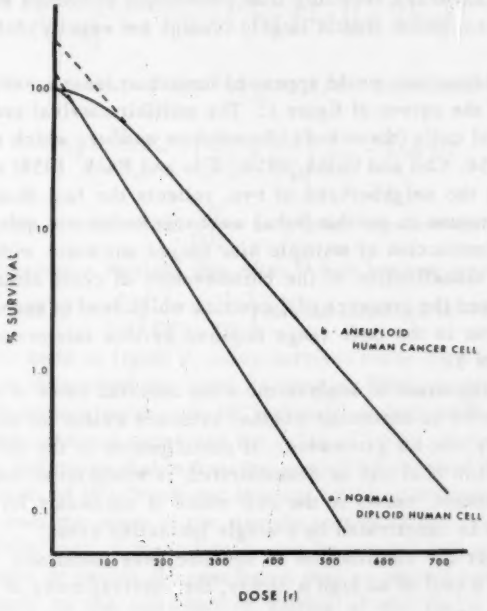

FIGURE 1. Survival curves of normal diploid (a) and aneuploid hyperploid (b) human cells. The S3 cell, of carcinomatous origin is a typical example of the latter (Puck and Marcus, 1956; Puck et al., 1957).

The first process would appear to be the main cause of reproductive death in the irradiation of haploid cells. Polyploid cells should not display any one-hit component in their radiation survival, unless non-chromosomal cell structures like the centriole are sensitive to one-hit inactivation processes. Diploid cells may be expected to exhibit survival curves to which all three processes might contribute, to varying degrees, as follows: A diploid cell containing recessive, lethal genes will contain a one-hit component in its survival curve, whose magnitude will be determined by the total volume of the genetic loci whose sister genes are recessive lethals. The two-hit specific mechanism will ordinarily contribute relatively little when an ionizing

radiation like x-ray, capable of breaking chromosomes, is employed, except in the special case where the chromosome number is very small. Otherwise the probability of death due to the third process will far outweigh the second, since a lethal chromosome restitution from breaks that can occur anywhere within the genome is much more probable than localization of hits at any two particular gene sites. By the same reasoning, cells of higher ploidy would exhibit still less susceptibility to single-hit processes, because each gene is protected by additional copies so that even occasional recessive lethals are less important. Similarly, the second process also becomes even less important than in the diploid case. Thus the main mechanism of reproductive death in polyploid cells would appear to be the formation of chromosomal complexes resulting from interaction of broken ends of different chromosomes, which should largely (though not exactly) follow a two-hit process.

These considerations would appear to furnish at least a semiquantitative explanation of the curves of figure 1. The multihit survival curve of S3 and other hyperploid cells (these have chromosome numbers which are hypotetraploid, Hsu, 1954; Chu and Giles, 1958; Tjio and Puck, 1958) which display hit numbers in the neighborhood of two, reflects the fact that these cells are virtually immune to one-hit lethal mechanisms but are quite susceptible to killing by interaction of multiple hits lodged anywhere within the karyotype. Direct visualization of the chromosomes of cells after x-irradiation has demonstrated the presence of dicentrics which lead to such chromosomal bridge formation in the dose range required by this interpretation (see, for example, figure 3).

It becomes important to analyze the x-ray survival curve of a highly polyploid cell like S3 to determine whether evidence exists for any appreciable contribution by one-hit processes. If participation in the killing curve by processes of this kind can be demonstrated, it would mean that some extrachromosomal target, exists in the cell which is necessary for reproduction, and which can be inactivated by a single ionization event.

If we neglect the contribution of specific gene mutations, which is certainly safe in a cell of so high a ploidy, the survival curve of figure 1 b can be represented by a formulation in which the surviving fraction, $\frac{N}{N_o}$, is the product of the probabilities of surviving each of the two possible lethal actions considered:

$$\frac{N}{N_o} = f_1(D) f_3(D) \tag{1}$$

where $f_1(D)$ is the one-hit process which is to be evaluated and $f_3(D)$ is the two-hit non-specific process which causes reproductive death whenever two or more effective events occur simultaneously in the same cell (Puck, 1959b).

$f_1(D)$ can be represented by e^{-D/D_1^o} where D is the dose in roentgens, and D_1^o is the mean lethal dose for all the one-hit processes that may be in-

volved. $f_3(D)$ is the probability that two randomly occurring breaks will interact to cause cell reproductive death, and can be approximately evaluated by the Poisson expression, $e^{-D/D_3^\circ}(1 + D/D_3^\circ)$ where D again is the dose and D_3° the mean dose needed to produce one of such interacting hits. (A more accurate representation of this term has been derived by J. Engelberg in this department, in an expression which explicitly contains the total number of chromosomes as a parameter (Engelberg, 1960).

Thus

$$\frac{N}{N_o} = e^{-D/D_1^\circ} \cdot e^{-D/D_3^\circ}\left(1 + D/D_3^\circ\right) \tag{2}$$

$$\text{Ln}\,\frac{N}{N_o} = -D/D_1^\circ - D/D_3^\circ + \text{Ln}\left(1 + D/D_3^\circ\right) \tag{3}$$

As the dose, D increases, so that $D/D_3^\circ \gg 1$, the expression becomes

$$\text{Log}\,\frac{N}{DN_o} = -\left(\frac{1}{D_1^\circ} + \frac{1}{D_3^\circ}\right)\frac{D}{2.3} - \text{Log}\,D_3^\circ \tag{4}$$

Therefore, if the function $\text{Log}\,\frac{N}{DN_o}$ is plotted against the dose, D, the curve should become a straight line after D has become reasonably large, with a slope equal to $-\frac{1}{2.3}\left(\frac{1}{D_1^\circ} + \frac{1}{D_3^\circ}\right)$ and an intercept of $-\text{Log}\,D_3^\circ$. Such a plot has been made in figure 2, using survival curve data for the S3 cell which have been determined for doses as great as 900r, at which the colony forming cells have been reduced to 10^{-6} of their original number. The curve obtained has the expected shape and the value of D_3° so obtained is 100r, a value which is indistinguishable from the overall D° obtained from the original survival curve, of 96r (Puck and Marcus, 1956). Similarly, the value of D_1° calculated from the slope of the straight portion of the curve comes out to be 720r. While each of these figures can be trusted only to about 10-20 per cent, the data do permit the conclusion that no one-hit process contributes significantly to the reproductive killing of the HeLa S3 cells by x-irradiation. It can also be concluded that single gene mutations, single chromosome breaks and one-hit damaging events to any other cell structures are rarely the cause of radiation death in these hyper-diploid cells.

Similar computation of the respective contributions by one-hit and two-hit processes to the x-ray survival curve of the normal diploid human cell is more difficult because of the lower precision of plating efficiency measurements in these cells. Improvement in the precision of these measurements is now developing and it is hoped before long to have more accurate data. At present it is clear that the apparent hit number is definitely less than two, and probably lies somewhere between one and 1.5. Refined analysis of this system may permit a simple means for determining the approximate proportion of recessive genes lethal to reproduction which may be present in any diploid cell.

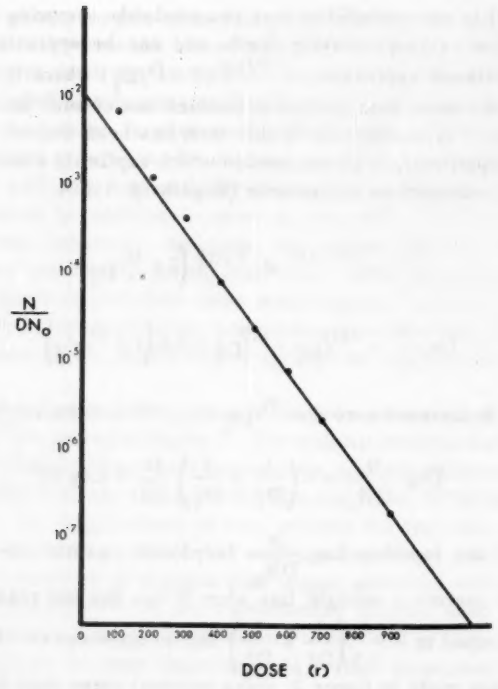

FIGURE 2. Plot of the function Log $\frac{N}{DN_o}$ against the dose D for S3 HeLa cell, as calculated from data of the survival curve as shown in figure 1b. As explained in the text, this function should become a straight line as the dose D increases, and from the slope and intercept of this line, values for D_1^o and D_3^o, the respective mean lethal doses for one-hit, and non-specific multiple hit processes can be calculated.

If the smaller hit number of the diploid cell as compared with cells of higher ploidy does indeed reflect the effect of recessive genes lethal to cell reproduction, one might expect that a diploid cell with no such defective genes would display a curve like that of the cells of higher ploidy. It is of interest, therefore, to examine the x-ray survival curve of cells of the inbred Chinese Hamster. This animal has been carefully bred by Dr. Yerganian (1959) through many generations of consecutive brother-sister matings, so that the resulting forms should by this time be predominantly homozygous. The survival curve of this cell which is primarily diploid, is indeed very much like the S3, with a hit number close to two (Puck, 1959b). While there are some complications which might prove later to prevent exact comparison of these two cell types—such as the smaller number and larger size of the Chinese Hamster chromosomes—it is encouraging that the results lie in the predicted direction.

The differences in slopes of the straight part of the survival curves of figures 1a and 1b would appear to reflect the operation of the following factors (Puck, 1959b):

a) The degree to which one-hit and multi-hit processes are respectively involved in the killing process, and the effective target sizes of each process. Thus, the effective target for one-hit processes would include all the genes vital for reproduction present in only a single copy within the cell. That for multiple-hit killing would include all the chromosomes capable of participating in lethal recombinations, or other kinds of cooperative interactions that could limit viability.

b) The probability of any given chromosome break contributing to a lethal recombination. This factor will be strongly influenced by the velocity of the reaction which reseals the broken chromosome ends. Thus, if this process occurs within a time that is very short compared to that of chromosomal movements, the most likely outcome will be a rejoining of the original break. If the reverse is true, the two ends will drift apart and the actual recombinations that occur will be completely random. Obviously, then, this parameter may be expected to vary strongly with the metabolic state of the cell and with the rate at which the radiation is administered.

II. DIRECT VISUALIZATION OF CHROMOSOMAL ANOMALIES IN X-IRRADIATED HUMAN CELLS

The chromosomal mechanism of mammalian cell killing deduced purely on the basis of survival curve analysis was substantiated when techniques for visualizing the karyotype of these cells were developed (Tjio and Puck, 1958). The kinds and frequencies of chromosomal aberrations which appeared after various doses of radiation were demonstrated to be in complete agreement with those expected theoretically, and established beyond doubt the exceedingly great vulnerability of mammalian chromosomes to damage by ionizing radiation (Puck, 1958).

In addition to furnishing support for interpretation of x-ray survival curves however, it is of great importance to establish quantitatively the dose dependence of different types of aberrations in order to gain understanding of the relationship between gene mutations and chromosome breaks in mammalian cells. However, the quantitation of chromosomal anomalies in irradiated mammalian cells is fraught with pitfalls, and at present the dose figures available can only be accepted with reservation, and probably as representing only the maximum doses needed to produce a chromosome break.

The first approximation to this figure was obtained by Bender (1957) who demonstrated that on the average 300r was needed to introduce one chromosome break per cell. Studies in our laboratory, in which attempt was made to overcome some of the parameters disturbing this picture, lowered this figure to the point where it is now less than one-tenth of the original estimate, and stands in excellent agreement with the data of the survival curves. The reasons for the tendency of the breakage dose to be overestimated are: a) Cells damaged by radiation exhibit a mitotic lag, and so may not be proportionately represented in the harvest of mitoses which is sampled at a later period. b) Many of the chromosomally damaged cells are doomed to an abortive mitosis, which can be observed only at particular

times which will be different from cell to cell. c) Uninjured cells, continuing their normal growth, contribute ever-increasing numbers of mitotic figures which tend to make the relative contribution of the chromosomally injured cells still smaller and finally d) chromosome breaks tend to become restituted at varying rates, so that the observed breaks represent merely those lesions which have not yet healed at the time of sampling. If such restitution takes place between fragments of different chromosomes, they may be recognizable, but in a great proportion of the cases, they obviously will be missed.

In a series of experiments designed at least partially to overcome these disturbing factors a value of about 40*r* was obtained as the dose required to produce an average of one recognizable chromosome break per diploid human cell (Puck, 1958). These data demonstrated the expected relationship that single-break processes increase approximately linearly with dose whereas multi-hit aberrations exhibit the expected initial threshold with virtually no anomalies of this kind appearing below 50*r*.

In these experiments, cells were fixed at various intervals after irradiation, and the number of simple chromosome breaks plus the number of recognizable abnormal recombinations were totalled for the entire series and averaged. In more recent studies attempt to improve this estimate has been made by taking into account that the number of visible chromosome breaks declines with time after irradiation because of normal and abnormal restitutions, only a fraction of which will be recognizable in later divisions. Hence, the average yield of hits (defined as a unitary chromosomal breakage process, which may or may not restitute) per r should decline as the incubation time is increased, although the manner of this decline is not readily predictable because of contributions from cells coming out of lag, which may continue for several additional days. The experimental results bear out this expectation. Hence, the most accurate value of the dose needed to produce an average of one break per cell per r would appear to be best estimated from the very first wave of mitoses after irradiation. The data so far available yield a value of about 20*r* as the mean dose producing an average of one visible break per cell. This dose appears to fit better with the observed D° value of 50*r* for these cells, since if the average chromosome breakage dose were actually equal to the latter figure, it would mean that a single chromosome break is sufficient to kill a normal human cell. This supposition is most unlikely particularly since we have demonstrated that human cells containing only 45 chromosomes, with the XO condition of the sex chromosomes, give single cell plating indistinguishable from that of normal cells with a full chromosomal complement (Tjio et al., 1959).

Figure 3b presents some of the typical single-hit breaks in diploid human cells which appear largely in the first mitoses following irradiation, and which increase approximately linearly with dose. Figure 3c demonstrates multi-hit complexes which arise through interaction of two or more single breaks, which appear in the later mitoses, and which exhibit a threshold dose as expected of multi-hit processes.

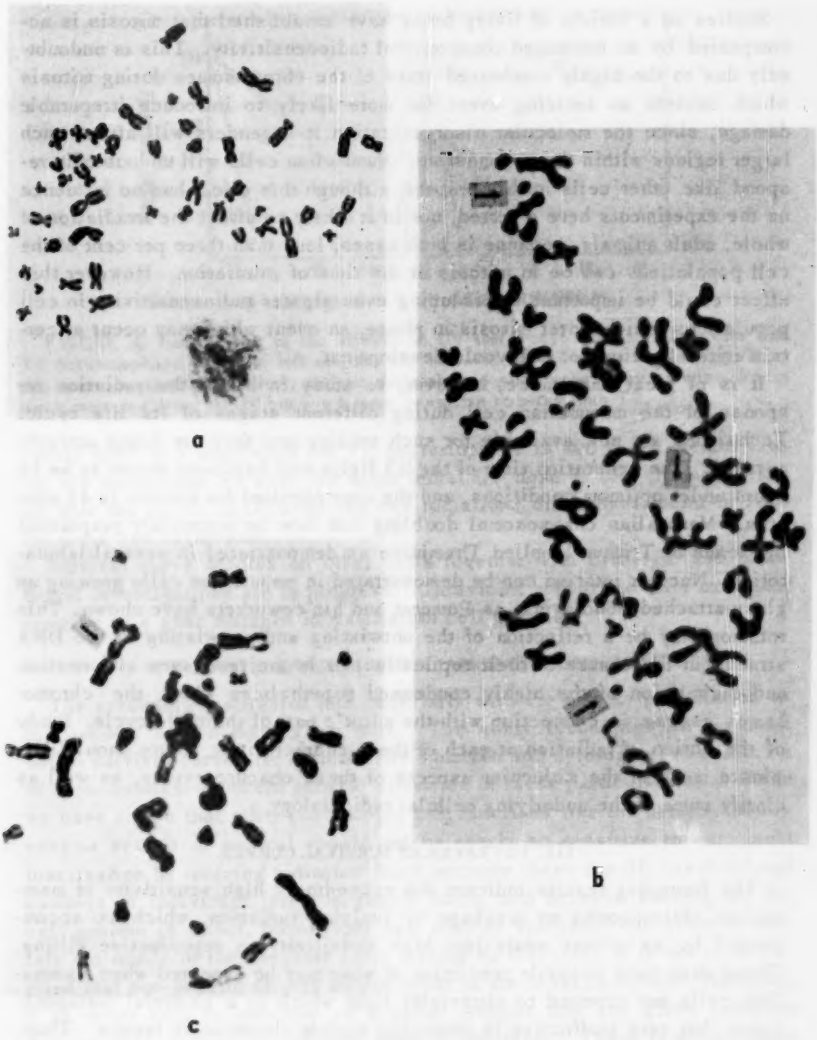

FIGURE 3. Typical chromosomal lesions produced by irradiation of normal human cells (Puck 1958, 1959b):

a) Unirradiated control.

b) Chromosome breaks which appear shortly after irradiation, but which rapidly reseal thereafter (50r). These breaks are approximately a linear function of the dose.

c) Example of an abnormal restitution of broken fragments from different chromosomes (150r). These aberrations become visible after the initial breaks have largely disappeared. As to be expected, these aberrant recombinations which require multiple chromosome hits, display a multiple-hit dependence on dose, with a threshold of about 50r.

Studies on a variety of living forms have established that mitosis is accompanied by an increased chromosomal radiosensitivity. This is undoubtedly due to the highly condensed state of the chromosomes during mitosis which renders an ionizing event far more likely to introduce irreparable damage, since the molecular disorganization it engenders will affect much larger regions within the chromosome. Mammalian cells will undoubtedly respond like other cells in this respect, although this effect has no influence on the experiments here reported, nor is it likely to affect the irradiation of whole, adult animals, because in both cases, less than three per cent of the cell populations can be in mitosis at the time of irradiation. However this effect could be important in producing even greater radiosensitivity in cell populations which enter mitosis in phase, an event which may occur at certain critical periods of embryonic development.

It is of great importance, however, to study in detail the radiation response of the mammalian cell during different stages of its life cycle. Techniques are now available for such studies and they are being actively pursued. The generation time of the S3 HeLa cell has been shown to be 18 hours under optimum conditions, and the time required for mitosis is 45 minutes. Mammalian chromosomal doubling can now be accurately pinpointed by means of Tritium-labelled Thymidine as demonstrated in several laboratories. Nuclear rotation can be demonstrated in mammalian cells growing as glass-attached monolayers, as Pomerat and his coworkers have shown. This rotation may be a reflection of the untwisting and re-twisting of the DNA strands in the course of their replication, or in the processes of formation and dissolution of the highly condensed superhelices which the chromosomes assume in connection with the mitotic part of their life cycle. Study of the action of radiation at each of these characteristic points should illuminate many of the molecular aspects of these obscure events, as well as clarify some of the underlying cellular radiobiology.

III. ULTRAVIOLET SURVIVAL CURVES

The foregoing results indicate the exceedingly high sensitivity of mammalian chromosomes to breakage by ionizing radiation, which is accompanied by an almost equivalent high sensitivity to reproductive killing. These data make possible prediction of what may be expected when mammalian cells are exposed to ultraviolet light which is a powerful mutagenic agent, but very ineffective in producing visible chromosome breaks. Thus, a highly polyploid cell like S3, which is killed primarily by multi-hit chromosomal aberrations like bridge-formation, should be exceedingly resistant to reproductive death by ultraviolet radiation, whereas normal diploid cells, and particularly those which already have an appreciable complement of recessive lethal genes, should be more sensitive. So far, only experiments with the S3 cells have been completed (Lee and Puck, 1958, 1959), and these results agree completely with the foregoing considerations. The results are shown in figure 4, in which the data for T2 bacteriophage are included as a standard of comparison. It is striking that whereas the mean

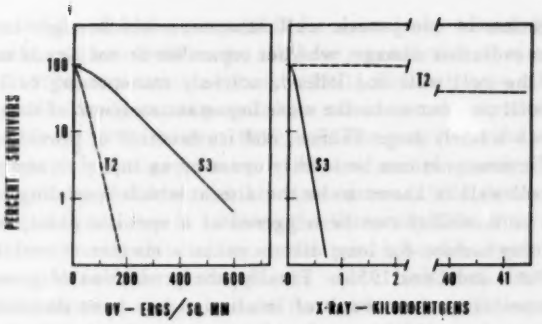

FIGURE 4. Comparison of the effects of UV and x-rays on S3 HeLa cells and T2 bacteriophage. On the left are the UV survival curves of the two materials in the same suspending medium, and on the right are shown the x-ray curves. S3 is much more resistant to UV but much more sensitive to x-ray than T2.

lethal dose of x-irradiation for T2 bacteriophage is 400 times greater than for the mammalian S3 cell, its mean lethal UV dose is four times smaller, so that the ratios of D^o's for the two radiations differ by a factor of 1600 for these two organisms.

Survival curve studies on other cells together with cytologic and mutational investigations are in progress. Ultraviolet appears to offer excellent promise as a gene mutagen in mammalian cell genetics.

IV. NON-GENETIC EFFECTS OF RADIATION ON MAMMALIAN CELLS

The preceding discussion has dealt only with the effects of radiation on the genome of the mammalian cell. The study techniques so far dealt with—survival curves for reproductive function and cytological examination of chromosomes—concern genetic activities of these particular cells. While we have shown that other physiologic cell functions like the activity of any enzyme system in general should not be nearly so sensitive to permanent inactivation by ionizing radiation[2] (both because these usually involve large numbers of individual interchangeable units, and because as long as the cell genome is intact, presumably new enzyme synthesis can occur to satisfy the needs of the daughter cells arising on division), it is not to be implied that non-genetic effects do not occur or are never important. However, it is never safe to conclude on *a priori* grounds that any given radiation damage does or does not involve genetic structures. There is even reason now to consider that the characteristic reproductive lag which has been demonstrated in mammalian cells as in other organisms (Puck and Marcus, 1956) may involve nuclear damage, rather than being exclusively cytoplasmic, and experiments testing this point are now under way in this laboratory.

[2]In this connection, it is worth noting that the depression of DNA synthesis following mammalian irradiation which at one time was considered a primary effect, now seems to be well explainable on the basis of several kinds of evidence as a consequence of reproductive failure resulting from chromosome damage (Puck, 1959a).

Consideration of non-genetic cell structures which might be maximally sensitive to radiation damage, whether reparable or not, leads to the possibility that the cell wall and indeed, actively transporting cell membranes generally, will turn out to be the most important members of this class. The cell wall has a fairly large volume, and its function of providing enclosure plus specific transport can be readily upset by an injury at any point. Moreover, the cell wall is known to be the site at which spreading reactions affecting its permeability can be triggered at a specific point, but travel in self-sustaining fashion for long distances in a manner resembling a chain reaction (Puck and Lee, 1955). Finally, the production of gross increases in cell permeability as a result of irradiation has been demonstrated in a variety of organisms (Billen et al., 1953). However, experiments performed so far have failed to demonstrate damage to mammalian cell wall permeability, even for doses much greater than the mean lethal dose. Thus S3 HeLa cells irradiated with 1300r which is 13 times the D° value for reproductive killing, continue to concentrate a dye like neutral red in their cytoplasm in a manner indistinguishable from that of unirradiated cells.

APPLICATION TO THE MAMMALIAN RADIATION SYNDROME

Attempts to apply results of single cell studies *in vitro*, to the extraordinarily complex situation attending irradiation of all or part of a mammalian body, must be attended with great caution. Yet the simplifications in understanding of some parts of the mammalian radiation syndrome which seem to have emerged from such attempts (Puck, 1959a), lend encouragement to this effort. While no one pretends that single cell studies will explain all or even most of the enormously complex phenomena which can be elicited in the animal, the desirability of exploring the usefulness of the simpler systems to the utmost is equally obvious.

It has been pointed out elsewhere (Puck, 1959a) how the ability of ionizing radiation to prevent sustaining reproduction of mammalian cells with a D° value in the neighborhood of 50–100r, provides a reasonable explanation qualitatively and in some cases at least semi-quantitatively for the following facts: 1) The mean lethal dose of whole-body radiation in mammals generally lies in the range of 400–600r. 2) Many of the symptoms of radiation injury express themselves only after a characteristic lag period has elapsed sometimes of days or weeks. 3) The tissues whose cells are reproducing most rapidly tend to exhibit the greatest radiation-induced pathologic changes. 4) Animals irradiated with doses close to the mean lethal range can be saved from death by administration of cells of the most rapidly dividing vital tissue, the bone marrow. Only viable cells are effective in this connection and these have been demonstrated to recolonize that organ and supply a new continuing supply of dividing cells. 5) Animals irradiated and then subjected immediately to low body temperatures fail to develop the symptoms of the radiation injury until the temperature is restored, after which the entire sequence of pathogenesis is initiated as though the irradiation had occurred at the time the temperature was raised. 6) Radioprotec-

tive agents which raise the x-ray MLD for animals by almost 50 per cent, exert a closely similar effect on the MLD for reproduction of single cells *in vitro* (Puck, 1959a; Morkovin and Puck, 1958). Similarly, the effects of O_2 on animal irradiation appear to parallel its effects on the survival curves of single cells (Gray, 1957; Dewey, 1959). 7) The very beautiful and comprehensive series of investigations on irradiated animals carried out over a number of years by Quastler and his associates (1956) demonstrated the profound effect of radiation in inhibiting cell reproduction to an extent that he was able to identify this action as the principle or at least one of the most fundamental lesions of the acute, whole body radiation syndrome. 8) The well-known fact that mammals can withstand much larger doses of whole body irradiation if these are fractionated over many weeks, is also immediately understandable by this picture: In the periods between irradiation, the cells still reproductively competent, multiply, so restoring their number, and neutralizing a large part of the effect of the previous dose. 9) Finally, the destruction of cellular reproductive potential through chromosomal damage in the region of 50-150 r also affords insight into the interaction of radiation with tumors and explains several of the quantitative aspects of tumor therapy (Puck, 1959b; Scott, 1958).

While the genetic damage to somatic cells appears able to provide a plausible picture for the phenomena here listed, certain manifestations of radiation in mammals may involve non-genetic cellular mechanisms. These processes probably include those actions which, unlike the preceding list, do not display appreciable lag periods which have been interpreted as the time needed for the affected cells to come to mitosis and thus make overt their latent chromosomal injury. An example of what may be such a non-genetic action is the ability of radiation to cause discharge of stored cell products such as the granules of the Paneth cells (Hampton and Quastler, 1956), or the phenomenon of radiation shock which can also occur shortly after exposure. These mechanisms deserve intensive study.

SUMMARY

The action of x-irradiation on mammalian cells has been studied under carefully controlled conditions *in vitro*, by means of single cell survival curves, and through direct visualization of the chromosomes after varying doses of radiation. Mammalian cells generally are exceedingly sensitive to destruction of their ability to reproduce, the mean lethal dose, D^0, for various animal cells varying between 50 and 150 r. The normal, diploid human cell exhibits a D^0 of 50 r, while that for aneuploid hyperploid human cells is about 100 r. A variety of independent kinds of evidence establish that the primary radiobiologic action responsible for reproductive death of human cells in these dose ranges is damage to the genetic apparatus, and primarily to the chromosomes.

Experiments demonstrate that a dose as little as 20-40 r is sufficient to introduce an average of one chromosome break per cell in normal diploid human cells *in vitro*. These breaks restitute normally if there is only one per

cell, and soon leave no visible evidence of their presence. If many breaks are introduced in each cell, abnormal recombinations such as dicentric chromosomes and other aberrations are formed. Analysis of the survival curves demonstrates that some cells, like the normal diploid human cell, are killed reproductively to a significant extent by single-hit events, but that hyperploid aneuploid cells require multiple hits, which produce complex chromosomal forms. The effects of ultraviolet radiation, which produces gene mutations but relatively few chromosome breaks, are readily explainable by these considerations. By means of the concept of chromosome breakage and the attending destruction of cell-reproductive function, it is possible to understand both qualitatively and quantitatively many of the features of the acute whole body radiation syndrome in mammals.

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CELLULAR RADIATION EFFECTS*

MAURICE ERRERA

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The knowledge of the physical nature of radiations has reached a degree sufficient to allow mathematical treatment of radiobiological problems. An example is the target theory; even if incorrect in its original form, it certainly has contributed to the understanding of biological effects of radiation. In this paper, however, we will use a biological approach based chiefly on biochemical arguments; the limitations in knowledge of fundamental biology will allow only a critical summary of a few facts plus, wherever possible, some generalizations from which new hypotheses might arise.

The understanding of cellular effects of radiation requires knowledge of where the primary chemical events occur and how they interact with each other to produce visible alterations of cells and organisms. Any hypothesis on the mechanisms of cellular effects of radiation should envisage: (a) growth (increase in weight of living matter), (b) cell division, (c) cell differentiation and morphogenesis, (d) mutation, (e) death, (f) recovery processes, and these phenomena should be understood at the level of individual cells (unicellular organisms, gametes, tissue cultures) and at the level of whole complex organisms.

The localization of initial damage at the level of cell organelles has been studied by cytological methods, and by biochemical investigations of fractions from disrupted cells. In spite of much effort, very few experimental data are available on what happens to tissues in the early stages, that is, within one hour or preferably less after irradiation.

EFFECTS OF RADIATION ON CYTOLOGICAL STRUCTURES

1. Mitochondria

a) *Oxidative phosphorylations.* Early inhibitions of oxidative phosphorylations have been reported in several instances (spleen, thymus; van Bekkum, 1955; Ord and Stocken, 1955). On the other hand, an increase in the incorporation of P^{32} into liver ATP has been observed soon after total body irradiation with 800 r (Sherman and Forssberg, 1954). This may have resulted from a decrease of ATP metabolism in muscle; in fact Albaum (1955) found a decrease in muscle ATP within two hours after irradiation.

Early effects of radiation on cell respiration in several animal tissues have been found, but not regularly. Simultaneous measurements of oxygen consumption and ATP formation revealed that high dosages were needed to

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inhibit respiration, which means that phosphorylations were uncoupled from respiration; the occasional findings of increased respiration may have resulted from this mechanism. It appears, thus, that there is at least in thymus and spleen, an early alteration of the complex electron and hydrogen transfer system responsible for phosphorylation. However, the mitochondria do not seem to be primarily affected because when they are irradiated *in vitro*, no inhibition has been noted (Potter and Bethel (1952), van Bekkum, Ord and Stocken, thymus or spleen), and no increased phosphorylation was found by Sherman and Forssberg with isolated liver. Moreover, there are some puzzling results by Fritz-Niggli (1955, 1956) who inhibited oxygen consumption in the presence of citrate or pyruvate in liver mitochondria prepared in hypoösmotic concentrations of mannitol with as little as 0.1r. These experiments may indicate that under osmotic strain irradiated mitochondria become defective; or, isotonic sucrose may have greater protecting efficiency than hypotonic mannitol. It would certainly be of interest to study irradiated mitochondria with the electron microscope; if preparations fixed in the routine manner show no early alteration, these might be made apparent if the mitochondria were put to a strain (osmotic or mechanical for instance), or if they were analyzed by the usual methods of mitochondrial analysis (Green, 1958).

An increase of adenosine-triphosphatase occurs in spleen shortly after doses of x-rays as small as 25 to 50r; however, since it occurs slightly later than the effect on phosphorylation (van Bekkum, 1955), the two effects appear to be independent. No increased adenosine-triphosphatase activity has been reported by Thomson, Tourtelotte and Cartarr (1952).

Secondary effects on mitochondria have recently been investigated by Ottolenghi, Bernheim and Wilbur (1955) who have shown an inhibition of choline esterase and succinoxidase by U.V.-irradiated preparations of nonsaturated fatty acids. Since the lipid structure of mitochondria may be involved in electron transport processes (Green, 1958) any alterations they could undergo might well result in alterations of function.

The illuminating work of Ephrussi (1953) and Slonimsky (1953) on yeast cells has led these authors to the idea that the level of respiratory enzymes in these cells depend both on cytoplasmic and on nuclear factors. The mutants "petite colonie," low in respiratory enzymes, are readily induced by U.V., the action spectrum indicating that nucleoproteins are concerned with the effect. The same mutants can also be obtained with x-rays, but in much smaller yield which could mean that the ribonucleoproteins involved are less sensitive to x-rays than to U.V. (Raut, 1955). The ploidy of the cells does not affect the yield of "petites" which may indicate the action to be chiefly cytoplasmic. Finally, as the original strain of yeast and the "petites" have similar survival curves with both x-rays or U.V., the killing by radiations is independent of the respiratory systems.

b) *Other mitochondrial functions*. Mitochondria also play a role in phospholipid synthesis. This function appears to be radioresistant. Phospholipids are possibly increased in the liver of irradiated mammals (Weinman,

Lerner and Entenman, 1956), not affected after radiation in the thymus (Thomson et al., 1952), and slightly diminished in the spleen (Cornatzer et al., 1954). It would be interesting to coordinate measurements of phosphorylations with those of lipid synthesis to find out whether the apparent different responses of both processes are due to experimental conditions or to different categories of particles.

2. Lysosomes

The most recently discovered cell particles are the lysosomes (DeDuve, 1954). These granules have been identified chiefly in the liver. They sediment along with the mitochondria in several fractionation processes. Their function has not been identified with certainty but they contain several types of hydrolytic enzymes: cathepsin, ribonuclease, deoxyribonuclease, acid phosphatase, β glucuronidase, sulfatase. Alterations by cold treatment, hypotonic media or detergents result in the liberation of all these enzymes. The fact that many of them (table 1) have been found to increase after irradiation may indicate a breakdown of these particles. Simultaneous determinations of several enzymes in irradiated tissue should clarify this hypothesis. Increase in cathepsin has been ascribed to the destruction by radiation of an inhibitor present in the blood, possibly in the leucocytes

TABLE 1

Possible lysosome enzymes inhibited by total body irradiation

Enzyme	Dose	Assay	Reaction	Remark	Author
Cathepsin					
spleen (rat)	...	24 h.	increase	...	Carter, 1949
liver (rat, mouse)	50 r	24 h.	increase		Ballin and Feinstein, 1952
liver (rabbit)	50 r	24 h.	increase	begins immed.	Feinstein and Ballin, 1953
Deoxyribonuclease					
liver (rat)	500 r	4–24 h.	inhib.		Douglass et al., 1954
spleen (rat)	500 r	4–24 h.	increase	...	Douglass et al., 1954
spleen (rat)	100–1000 r	...	none	...	Carter, 1949
liver, spleen (rat)	850 r	immed.	increase	acid DNAse	Goutier-Pirotte, Goutier, 1959
spleen (mice, marrow)		immed.		acid & alcaline DNAse (inhibition of enzyme inhibitor suggested for alcaline DNAse)	
β glucuronidase					
lymphatic (rat)	200–600 r	4 h.–15 days	increase	correlation with tissue atrophy	Pellegrino and Villani, 1957
Ribonuclease					
liver (rat)	600 r	24 h.	increase	lasts for approx. 8 days	Douglass et al., 1954
liver (rat)	600 r	2 weeks	increase	...	Roth et al., 1953
spleen (rat)	100 r	24–48 h.	no effect	...	Carter, 1949

(Feinstein and Ballin, 1953) and liberation of alkaline deoxyribonuclease to destruction of a DNAse inhibitor (Kurnik et al., 1958). If the physiological functions of these inhibitors was to protect the integrity of the lysosomes, then the lysosome and the inhibitor hypotheses would be compatible. This hypothesis would certainly not help to elucidate the increase of other enzymes such as adenosine triphosphatase (Ord and Stocken, 1955) which do not appear to be bound to lysosomes.

3. Microsomes

In vivo microsomes are probably bound to the endoplasmic reticulum. There may be several classes; those linked to cholesterol synthesis which retain their biochemical activity after ribonuclease treatment (Bucher and Garrahan, 1955) also seem to be radioresistant. The smaller microsomes play an important part in protein synthesis; they are the site where amino acids are organized after they have been "bound" to soluble ribonucleic acid molecules (see Zamecnik, 1958). No radiobiological studies on the different steps of these processes have been performed. On the whole proteins seem to be formed in the early post-irradiation period, often at increased rates: this is known for globin and hemin synthesis in bone marrow or spleen (Richmond, Altman and Salomon, 1951) and for hemin in reticulocytes (Nizet et al., 1954). Part of this last effect was obtained when reticulocytes were incubated with plasma irradiated *in vitro*. The inhibition of incorporation of iron into hemoglobin only occurs at a later period (Suit, Lajtha, Ellis and Oliver, 1955). The later decrease of synthesis of globin and of hemin may result from the inhibition of cell division or of cell differentiation.

Pancreatic amylase, trypsin and lipase are all inhibited 12 to 36 hours after 400–600 *r* total body irradiation (Rausch and Stentstrom, 1959) but amylase formation in mouse pancreas incubated *in vitro* 24 hours after irradiation with 2000 r was normal (Hokin and Hokin, 1956). These conflicting results could be due to different experimental conditions; the inhibition of protein synthesis in the whole animal might be a result of the liberation of ribonuclease by disrupted lysosomes, or the late decrease in protein synthesis could be secondary to nuclear damage. It has been shown in very informative enucleation experiments by Brachet (1957) that one of the most striking consequences of the removal of the nucleus in amoeba is a slow loss of cytoplasmic ribonucleoproteins. We shall come back to this problem.

No direct evidence of damage to microsomes can be deduced from the behavior of individual enzymes. In the liver 50 per cent of the esterase is bound to these particles; it is not changed after 500 r. Of other enzymes bound to liver microsomes (DPNH TPNH cytochrome reductase, glucose-6-phosphatase), no radiation responses are known. Spleen DPN cytochrome reductase does not seem to be affected (Eichel, 1957). Glucose-6-phosphatase is increased in salivary glands after irradiation.

4. Cytoplasmic soluble components

One can make no generalizations concerning soluble components of the cytoplasm. Many proteins are radioresistant *in vivo*, even if sulfhydryl groups are necessary for function. Glycolysis is inhibited in tumors as shown in early experiments of Crabtree (1936) and Holmes (1937) but is not inhibited in rat spleen two to five hours after 700 *r* (van Bekkum, 1954). In yeasts, Sherman and Chase (1954) have shown that glycolysis becomes sensitive if the microorganisms had been grown on low nitrogen media; the effect becomes measurable only two hours after irradiation. The interpretation of such experiments is not easy; low nitrogen content may mean low concentrations of intracellular protecting agents, lower enzyme concentrations or a greater percentage of deterioration of enzymes during their functioning.

5. Cell membrane

Decreased utilization of a substrate could result from its slower penetration into the cell, possibly resulting from an impairment of the transport enzymes on the cell surface. The retardation or sometimes inhibition of induced enzyme synthesis as a result of x-irradiation could be due to an inhibition of permease formation (Monod, 1956) on the surface of the cell. The late inhibition of respiration of glucose described by Billen, Stapleton and Hollaender (1953) in x-irradiated *E. coli*, could also be interpreted in this manner. If the bacteria are *preincubated* without glucose at 37°, then the respiratory deficiency becomes immediate, probably because all the endogenous substrate has been consumed and the penetration of new substrate possibly retarded. Different results are obtained with other substrates (succinate, pyruvate), but much less appears to be known about the penetration of these molecules through cellular membranes than is known for glucose. Besides, the oxidative pathways of these substrates are shorter than those of glucose.

One method of testing whether an enzyme is located on the surface of a cell would be to study its possible protection by non-diffusible agents: if the active groups of an enzyme are turned toward the medium, it should be protected by radiosensitive macromolecules, against indirect radiation effects. The inhibition of respiration of "dilute" suspensions of spermatozoa, chiefly when substrates necessitating SH enzymes are used (Barron, Gasovda and Flood, 1949; Barron and Seki, 1952) could be due to such an indirect effect upon a surface enzyme. A concentrated suspension is much more resistant, both with respect to respiration and fertilizing power (Evans et al., 1942); in the latter case, protection by ovalbumin, glycylglycin or by seminal fluid has been described. The experiments of Sherman and Chase (1949) have also shown that the effect on glycolysis was greater for more dilute suspensions. Such effects of radiation on enzyme systems which depend on the *concentration* of the cells during irradiation might therefore be due to a competition of surface enzymes for free radicals. Of course the

concentration effect could be also the consequence of anoxic conditions arising in the most concentrated suspension; however, an oxygen effect should also affect the inhibition of cell multiplication, and there is no influence of the cell concentration with respect to this function. Hence, a membrane phenomenon might be worth thinking about (another example will be cited later).

6. Nuclear phenomena

The most important forms of nuclear radiation damage are the chromosome aberrations; they have been discussed in other papers of this symposium. Lesser chromosome damage leads to point mutations which, like the more visible aberrations, are not definitively established immediately after irradiation treatment and can therefore be modified after the primary lesions have been established (Witkin, 1956; Doudney and Haas, 1958; Luning and Hannertz, 1957).

Other aspects of nuclear damage may be mentioned:

a) Nuclear phosphorylations are highly radiosensitive, a total inhibition following doses of the order of 100 r (Creasy and Stocken, 1957). Since the incorporation of radioactive substrates are only partly inhibited by these doses, one may be able to find a correlation between nuclear phosphorylations and the specific protein or nucleic acid fraction concerned, as it is known that different protein or nucleic acid fractions of thymus nuclei incorporate at different rates.

b) Incorporation of amino acids into proteins, and of purine bases or pyrimidine nucleosides into nucleic acids in the isolated nuclei of thymus, appendix or liver cells is inhibited by doses of the order of 300 r (Ficq and Errera, 1958; Logan, Ficq and Errera, 1959; Logan, 1959). In the case of amino acid incorporation at least, one is presumably dealing with synthetic processes (Mirsky, Osawa and Allfrey, 1956).

c) Nothing can be said about specific nuclear enzymes such as those concerned with coenzyme synthesis or the metabolism of uridine nucleotides (Herbert et al., 1955).

d) Brachet has shown deficiencies of ATP levels in non-nucleated amoebae, in lethal hybrids, and in embryos obtained from eggs fertilized with nitrogen mustard-treated sperm. They appear in anaerobic conditions, and (in the embryos) are associated with conspicuous nuclear anomalies (abnormally high or low Feulgen staining, abnormal nucleolar basophilia). In contrast, under aerobic conditions ATP was not used as rapidly as in control organisms. This seems to indicate a nuclear function related to anaerobic phosphorylations which should be considered in radiobiology.

e) Little is known about specific nucleolar lesions; they have been reviewed elsewhere (Errera, 1957).

EFFECTS OF RADIATION ON BIOLOGICAL FUNCTIONS

Having reviewed the effects of radiations on these various cellular processes, one must attempt to understand their relations to the known biologi-

cal effects of irradiation. We shall not attempt to review all important biological functions which radiation can affect, but limit our discussion to a few only.

A previous study of nucleo-cytoplasmic relationships in irradiated cells (Errera et al., 1958) led to the conclusion that the cytoplasm is not necessarily less sensitive than the nucleus, but the latter has a greater probability of expressing damage through its function of regulating many essential processes. In resting cells, cytoplasmic damage may become relatively much more important than in dividing cells. There are, however, indications of cytoplasmic recovery even when the nucleus has been damaged, although the nucleus is probably essential for such a recovery; an effect of normal cytoplasm on nuclear damage seems less obvious from Iverson's (1958) U.V. data. In general, however, it seems certain that cytoplasmic integrity, necessary to provide the nucleus with essential substrates, must play a role in nuclear recovery processes; but these inter-relationships still need to be studied.

1. Effects of radiation on growth

Many bacteria grow into "snakes" after U.V. or x-irradiation; barley seeds give bigger sprouts when they have been subjected to x-rays (Moutschen, Bacq and Herve, 1956); tissue culture cells grow into polyploid giants (Puck and Marcus, 1956). High doses of x-rays do not prevent and sometimes even enhance growth and regeneration of whole or enucleated Acetabularia (Bacq et al., 1957). Such experiments have led to the concept that low dosages of radiations may "stimulate" cells. In Moutschen's experiments where growth of irradiated grains of barley was enhanced, mitoses were inhibited and the cells were simply elongating. The increase in elongation could be due to the greater availability of low molecular substrates, such as ATP, for protein or cellulose synthesis which would have been used for mitotic processes in unirradiated controls. It is probable that instances of increased synthesis (lipid and glycogen in the liver and intestine—Coniglio, McCormack, Hudson, 1956) could be interpreted in a similar way.

2. Cell division

One of the major effects of radiation is the inhibition of mitosis. In microorganisms, tissue cultures and gametes, there is often retardation at first, then divisions go on, but are finally stopped. This occurs during differentiation in the case of embryos obtained from irradiated gametes.

Cell division is known to depend on several factors:

a) *Deoxyribonucleic acid* synthesis appears to be necessary for cell division; so far, no evidence has been found for cell divisions taking place without preliminary DNA synthesis except for the case of cytoplasmic division in non-nucleated embryonic cells (see Brachet, 1957). In microorganisms, deprivation of a specific precursor of DNA blocks nuclear division and leads to the formation of elongated cells (Thermobacterium—Jeener and Jeener, 1952; *E. coli*—Cohen, 1957). However, a block in DNA syn-

thesis is not the only factor leading to inhibition. In experiments of Gaulden (1956) and Carlson (1954) on grasshopper neuroblasts, mitosis was delayed by irradiation given before the beginning of prophase but after the DNA for this had been synthesized. In U.V. treated *E. coli*, Deering (1958) has shown that cytoplasmic cleavage could be inhibited with doses which do not affect DNA synthesis. Data by Howard and Pelc (1953) on Vicia roots lead to similar conclusions.

The cause for the inhibition of DNA synthesis is not yet clear. Work on U.V.-irradiated bacteria has shown that the low molecular precursors of DNA were present and had accumulated during the time DNA synthesis was blocked (Kanazir and Errera, 1956) and these findings have been found to apply to x-irradiated spleen (Becarevic, 1957) and thymus (Ord and Stocken, 1958). Therefore, it is either the formation of deoxynucleoside triphosphates or the actual duplication of the double helix which is inhibited.

DNA of yeasts becomes more susceptible to alkaline treatment after x-irradiation (Chantrenne, 1959; Meissel, 1955) and dissociation of spleen deoxyribonucleic is facilitated (Cole and Ellis, 1957). This could result from digestion of the protein moiety by increased proteases, the diffusing nucleic acid being perhaps responsible for the accumulation of basophilic material appearing in the neighborhood of the nucleus of spleen, lymph nodes, intestinal epithelium or tumor cells observed by cytochemical procedures (Mitchell, 1942).

Remarkable experiments on bacteriophages point to the possibility that x-irradiation causes breakage affecting both strands of the DNA double helix whereas U.V. data can best be interpreted on the assumption that only one of the two helices is disrupted at each event (Stent, 1958).

Calculations of the expected number of ionizations in DNA molecules lead to the conclusion that even with low doses of x-rays (100r) several ionizations could occur in DNA (Guild, 1957). From work of Schramm (1959) on Tabacco mosaic virus, the loss of one single *amino group* by the action of nitrite can lead to mutations.

Stent (1958) compared inactivation of bacteriophage by x and U.V.-irradiation and by P_{32} decay and concluded that the information carried by phage DNA might be transmitted to the new DNA through an intermediary protein template. That *protein synthesis* is necessary for DNA synthesis has been demonstrated for bacteriophage replication in amino acid deficient hosts (Burton, 1955) and for chloramphenicol-treated *E. coli* (Hershey, 1957). Harold and Ziporin (1958) and Drakulic and Errera (1959) showed in nitrogen mustard-treated and in U.V.-irradiated bacteria that a block in DNA synthesis which is normally only temporary becomes irreversible if protein synthesis is inhibited by chloramphenicol. During this period, the RNA fraction containing most of the bacterial DNA (the lighter fraction obtained by centrifuging osmotically shocked protoplasts of *E. coli*) appears to lose to the supernatant fraction part of the RNA formed after irradiation (Drakulic, 1959). This could result from an inhibition of synthesis of a small protein

fraction leading to the formation of soluble RNA instead of insoluble ribonucleoprotein (Pardee, 1956; Neidhardt and Gros, 1957).

This *ribonucleoprotein* could in turn be responsible for the formation at each mitotic cycle of new enzymes like transphosphorylase or DNA polymerizing enzymes, which if inhibited either directly by radiation or by the absence of the specific ribonucleoproteins would cause an inhibition of cell division.

In grasshopper neuroblasts microirradiation (U.V. microbeam) of the nucleolus between the end of synthesis of DNA and early prophase inhibits cell division irreversibly (Gaulden and Perry, 1958). The function of the nucleolus during mitosis is not yet clear and what happens to its nucleoproteins is still unknown, but the nucleoli appear to be formed in each daughter cell from special regions of chromosomes. The fact that ribonuclease also inhibits cell division (Brachet, 1957) may be related to this nucleolar function—but this of course is speculation.

b) *Cytoplasmic division*, as already stated, can be blocked although overall synthesis has not been inhibited; and mechanisms of new cell wall formation, of spindle formation and division, and of its possible role of distributing ribonucleoproteins to the daughter cells are still very poorly known. The role of SH groups becoming oxidized as spindle proteins become organized has been postulated by Rapkine (1931), and Brachet (1957) and recent experiments of Mazia have confirmed these views. Nickerson and Falcone (1956) have suggested a possible role during cell division of protein disulfur reductase, which reduces —S—S— bonds present in the membrane of microorganisms. It would be of interest to look for similar systems in other cells. The interference of radiation with SH proteins has been put forward by Barron about a decade ago, but this theory has lost favor. Early effects of ionizing radiation on SH compounds *in vivo* have been found negligible in recent years. This does not mean, however, that all SH proteins are resistant, and that their sensitivity could not vary with the physiological condition of the cell; certain SH groups may be particularly vulnerable owing to their low oxido-reduction potential. Other mechanisms related to the inhibition of cell division will certainly spring up as our knowledge of the normal processes becomes more accurate and better defined biochemically.

3. Cell movements

Cell motility has so far been very much neglected in radiobiology; owing to its great importance as a factor in embryonic differentiation this cellular function should not be overlooked.

Prevention of sperm motility may be the result of the inhibition of oxidative phosphorylations as shown by Kanazir and Errera (1955) for U.V.-irradiated frog spermatozoa; high x-ray doses are needed for this effect in the mammal (Cassarett and Cassarett, 1957; Evans et al., 1942).

Movements of the cell surface related to pinocytosis and to phagocytosis might be worth studying.

4. Cellular differentiation

The highly complex process of differentiation should be analyzed at different degrees of cellular complexity.

a) *Induced enzyme formation in microorganisms.* U.V. light appears to be much more effective than x-rays if compared on the basis of killing efficiency. As the U.V. action spectrum is that of a nucleoprotein it has been suggested that the target is a cytoplasmic nucleoprotein (microsomes ?) which would be more sensitive to U.V. than to x-rays since RNA absorbs about 50 per cent of the total energy of U.V. light but only a few per cent of the x-ray energy absorbed. However, the difference between x-ray and U.V. effects is rather difficult to express quantitatively: U.V. absorption of cells can vary considerably with the experimental conditions, and the killing efficiency can differ in strains which respond similarly when adaptive enzyme formation is studied. The case of *E. coli* B and B/r is extremely suggestive: to produce a 20 per cent inhibition of galactosidase induction one needs a certain dose of irradiation, which with *E. coli* B leaves only .00006 per cent survivors (a picture very similar to that usually found with x-rays) while with the B/r strain there are still 0.3 per cent survivors. Killing and inhibition of enzyme induction are not equally affected by *cell concentration* during irradiation: there is no effect of dilution on survival, but a striking one on adaptation; in the latter case, U.V. is more efficient for dilute suspensions. This could be a surface phenomenon: if the enzyme inhibited is a permease, then diluting would probably give a greater percentage of U.V. energy on each cell surface, whereas a nuclear effect could not be expected to vary at cell concentrations where all U.V. is absorbed (Kaplan, Rosenblum and Bryson, 1953). One could also interpret some of the x-ray data by assuming that the retardation of enzyme formation (Baron, Spiegelman and Quastler, 1953) is due to a lag in the penetration of the substrate into the cell (inhibition of permease formation), the intracellular process of induction being resistant since the rate of increase of the intracellular enzyme is not slowed down.

Finally, and this has only been studied with U.V., the induction process is much more sensitive when the irradiation is given *before* the inductor substrate; the resistance of the system increases gradually with time during induction (Swensson and Giese, 1950; Torriani, 1956), the completely formed enzyme being radioresistant.

Chantrenne and Devreux (1959) have shown that during the induction of catalase by oxygen in yeasts cultured previously in anaerobiosis, there is a small ribonucleic acid fraction metabolizing rapidly; this metabolism is inhibited by U.V. as is the induced catalase formation (Becarevic, 1957). In contrast, high doses of x-rays, although they completely inhibit budding and DNA metabolism, provoke a very notable increase of activity of this RNA fraction as well as increasing enzyme formation.

In induced synthesis in microorganism, nuclear functions do not appear to play a great role although not all strains of a given bacterial species are

inducible: and as shown by Chantrenne and previously by Baron, Spiegelman and Quastler (1953), complete inhibition of DNA metabolism does not affect the speed of enzyme formation but only retards it.

Chantrenne and Devreux (1958) also showed that in yeasts grown anaerobically which have lost most of their normal respiratory enzymes, x-irradiation induces the formation of catalase, as would hydrogen peroxide or oxygen; this may be important evidence in favor of the formation of peroxides during irradiation. As determinations of enzyme activity were done on whole cells as well as on homogenates or autolysates, it seems unlikely that the phenomenon is due to a liberation of preformed catalase as suggested in experiments by Kaplan (1955). Cytochrome C peroxidase appears to behave like catalase (Sels, 1959).

b) *Morphogenesis of unicellular organisms.* The case of *Acetabularia mediterranea* has been studied by Hämmerling and by Brachet and their coworkers. With respect to survival non-nucleated fragments of this alga are more sensitive to x- and U.V.-irradiation than the nucleated rhizoid; when the whole alga is irradiated, one usually sees the cytoplasm cytolyze and regenerate from the nucleated part even though both have received the same dose of irradiation. If *morphogenesis* is studied it is seen that *U.V.* inhibits it whether a nucleus is present or not, but x-rays on the contrary *increase* the number of caps (Bacq et al., 1957) as a simple enucleation does (Brachet, 1957). It is possible that the nucleus competes with the cytoplasm for certain metabolites which become available to the cytoplasm if nuclear functions are inhibited. The radiosensitive processes in this case might be those responsible for the formation of "morphogenetic substances" inside the nucleus, because when whole algae are x-rayed one observes a late inhibition of cap formation. As Six (1956) has shown that the U.V. action spectrum for the inhibition of morphogenesis is that of a ribonucleoprotein (there is no measurable DNA in these algae), these morphogenetic substances are probably ribonucleoproteins, which, once formed, would be sensitive to U.V. but no longer to x-rays, like those responsible for induced enzyme synthesis in microorganisms.

The case of the formation of the gullet in Paramecia has been studied with the aid of U.V. microbeam irradiation (Hanson, 1955). The new gullet is formed from an anlage originating on the right side of the pharynx. Irradiation of the gullet *before* the formation of the anlage retards considerably not only the formation of the new gullet but also the division of the parameciae. Hanson (1955) has shown that irradiation at a later time leads to anomalous gullet formation of a degree the less pronounced the later one irradiates. It seems that *predifferentiation stages* are quite generally more sensitive than the actual process of differentiation.

c) *Induced synthesis in higher organisms.* Very little is known about induced synthesis in multicellular organisms, although it is probable that the formation of new enzymes, as embryonic development progresses, has much in common with induced synthesis in less complex organisms. However, in complex organisms like mammals, the processes are complicated by hor-

monal influences and cannot easily be dissociated from cell division. The only instance studied is that of tryptophane peroxidase, the synthesis of which in mammalian liver can be induced by hydroxycortisone (Knox, 1955) as well as by high concentrations of tryptophane (Gros et al., 1954); the second inducer only is capable of acting on liver *in vitro*. Total body irradiation increases the formation of the enzyme in a few hours, but *not* in adrenalectomized animals. However, if the *induction* with tryptophane is studied, 400r *inhibits* enzyme formation after two-three days and also the incorporation of valine into the liver fraction containing the enzyme. It is probable that the hepatic system necessary to synthesize *new* enzyme is inhibited but that this inhibition is masked during the first few days following irradiation on account of the already existing endocrine stimulation.

c) *Embryonic development.* It would certainly be of great interest to analyze this process of differentiation further, because, although the inhibition of cell division is certainly one of the causes of the inhibition of differentiation in complex organisms, it may not be the only one. For instance, embryos formed from a normal and an irradiated gamete stop dividing in early gastrula when embryonic differentiation begins. As cleavage has been going on through many division cycles, the mitotic mechanisms seem to be working normally. Does division stop because uneven distribution of chromatin to daughter cells through several cycles will lead to deficiencies which will become manifest because precisely at this stage new types of proteins are being formed under the influence of natural inducers and organizers? Is the primary lesion necessarily in the chromatin as it almost certainly is when the sperm has been irradiated, or is there room for cytoplasmic abnormalities in the case of irradiation of the egg cell or the developing embryo? How would a temporary inhibition of oxidative phosphorylations or of cellular movements affect a developing embryo, when everything is timed with such great precision? What would be the effect of a local release of nucleolytic enzymes, or a very temporary inhibition of exchange mechanism at the surface of a cell?

We do not want to minimize the importance of nuclear damage of the type which has been so beautifully clarified by Hertwig (1911), Dalcq and Simon (1932), Muller (1954), the Russells (1954), but we want to stress the existence of other damages which could profoundly affect the evolution of chromosome lesions or even by themselves cause abnormal cellular behavior at a time when coordination of cell movements and metabolism is indispensible for normal development.

SUMMARY

A critical review is given of radiation effects on cytological structures: mitochondria, lysosomes, microsomes, cytoplasmic soluble components, cell membranes, and nucleus. The implications of these changes for the analysis of radiation effects upon biological functions are discussed. The functions studied are cell growth, cell division, movements and differentiation.

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